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(54) Title: COMPOSITIONS CONTAINING POLYETHYLENE GLYCOL AND USES THEREOF

(57) Abstract

The present invention provides therapeutic compositions containing polyethylene glycol, methods for making the compositions, and methods of use thereof.

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COMPOSITIONS CONTAINING POLYETHYLENE GLYCOL AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. provisional Patent Application Serial No. 60/056,744, filed August 10, 1997; the disclosure of which is hereby incorporated herein by reference.

FIELD OF INVENTION

This invention relates to therapeutically effective compositions of matter containing polyethylene glycol (PEG) and methods of use thereof. More specifically, it relates to compositions containing PEG that exhibit antiapoptotic activity and methods of using compositions containing PEG to inhibit apoptosis or to protect, preserve or restore cell, tissue or organ function.

BACKGROUND OF THE INVENTION

A wide variety of physiologic damage is due to cell death. Two forms of cell death, necrosis and apoptosis, have been described and are being intensively and widely investigated. Kerr et al. (1972) *Br. J. Cancer* 26:239–257; Umansky (1996) *Molekulyarnaya Biologiya* 30:285–295; and Vaux and Strasser (1996) *Proc. Natl. Acad. Sci.* 93:2239–2244. Necrosis is generally considered to be a result of severe irreversible cell damage. It is characterized by early swelling of the cell and its cytoplasmic organelles with subsequent rupture of the cellular membrane.

Apoptosis.

Apoptosis is a normal physiologic process that leads to individual cell death. This process of programmed cell death is involved in a variety of normal and pathogenic biological events and can be induced by a number of unrelated stimuli. Changes in the biological regulation of apoptosis also occur during aging and are responsible for many of the conditions and diseases related to aging.

Studies of apoptosis have implied that a common metabolic pathway leading to apoptosis can be initiated by a wide variety of signals, including hormones, serum growth

factor deprivation, chemotherapeutic agents, ionizing radiation, and infection by human immunodeficiency virus (HIV). Wyllie (1980) *Nature* 284:555-556; Kanter, et al. (1984) *Biochem. Biophys. Res. Commun.* 118:392-399; Duke and Cohen (1986) *Lymphokine Res.* 5:289-299; Tomei, et al. (1988) *Biochem. Biophys. Res. Commun.* 155:324-331; Kruman, et al. (1991) *J. Cell. Physiol.* 148:267-273; Ameisen and Capron (1991) *Immunol. Today* 12:102-105; and Sheppard and Ascher (1992) *J. AIDS* 5:143-147. Apoptosis can also be induced by mild, non-catastrophic cell injury and can be concomitant with adjacent necrosis. Agents that affect the biological control of apoptosis thus have therapeutic utility in numerous clinical indications.

Apoptotic cell death is characterized by morphologic changes such as cellular shrinkage, chromatin condensation and margination, cytoplasmic blebbing, and increased membrane permeability. Gerschenson et al. (1992) *FASEB J.* 6:2450-2455; and Cohen and Duke (1992) *Ann. Rev. Immunol.* 10:267-293. Specific internucleosomal DNA fragmentation is a hallmark for many, but notably not all, instances of apoptosis.

Several genes and gene families involved in signal transduction and modulation of apoptosis have been described. By definition, necrosis can be prevented only by decreasing cell injury. Apoptosis, however, is an active cellular response to a physiologic or external signal and can be modulated by interfering with the apoptotic pathway. Prevention of apoptosis by up-regulation of bcl–2 and bcl–x expression or prevention by inhibitors of ICE-like proteases are typical examples of this approach to modification of cell death. Umansky (1996); Vaux and Strasser (1996); Nunez et al. (1994) *Immunol. Today* 15:582–588; and Whyte (1996) *Trends in Cell Biol.* 6:245–148.

Apoptotic cell death appears to play a significant role in the tissue damage that occurs in association with, *e.g.*, ischemia, organ transplantation, and various gastrointestinal disorders.

Ischemia and reperfusion.

Ischemia is the result of decreased blood flow to a particular area or organ of the body. Ischemia can result in several important types of physiologic damage to the organs, tissues and cells of the body, including, without limitation, tissues and cells of the brain, spinal cord and heart.

Myocardial ischemia is the result of an imbalance of myocardial oxygen supply and demand. The term "myocardial infarction" describes the irreversible damaging of the

cells as well as the necrosis occurring as a consequence of a total or important reduction of the coronary flow feeding some areas of the cardiac muscle; moreover, it may be a consequence of an insufficient increase of the coronary flow with reference to an increased requirement of oxygen, as can happen under some conditions of stress.

The most important consequence of acute myocardial ischemia is the death of individual heart cells, which leads to organ dysfunction. Early reperfusion decreases heart damage; however, massive cell death can occur with the restoration of blood flow. In this instance, the cells that die are from the group of cells that were still alive at the end of ischemia. Karmazyn (1991) *Can. J. Physiol.* 69:719-730; and Fox (1992) *Cardiovasc. Res.* 26:656-659.

Recent data indicate that apoptosis plays a significant role in heart injury induced by ischemia and subsequent reperfusion. Gottlieb et al. (1994) *J. Clin. Invest.* 94:1621-1628; Umansky et al. (1995) *Cell Death and Differentiation* 2:235-241; Umansky et al. (1996) *Basic and Applied Myology* 6:227-235; and Itoh et al. (1995) *Am. J. Pathol.* 146:1325-1331. Severe cell damage during prolonged ischemia appears to result in necrotic death of myocardial cells. However, if the ischemia is relatively limited in extent and duration, the apoptotic pathway is initiated. Restoration of blood flow (reperfusion) allows apoptosis to proceed. Insulin-like growth factors (IGF) and calpain inhibitors, which are capable of preventing apoptosis in different systems, also inhibited apoptosis of cardiomyocytes following ischemia and reperfusion both *in vivo* and *in vitro*. Umansky et al. (1995); and Buerke et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:8031-8035.

Cardiovascular disease is the leading cause of death among Americans. The development of new therapeutic agents capable of limiting the extent of myocardial injury, i.e., the extent of myocardial infarction, following acute myocardial ischemia is a major concern of modern cardiology. The advent of thrombolytic therapy during the last decade demonstrates that early intervention during heart attack can result in significant reduction of damage to myocardial tissue. Large clinical trials have since documented that thrombolytic therapy decreases the risk of developing disturbances in the heartbeat and maintains the ability of the heart to function as a pump. This preservation of normal heart function has been shown to reduce long-term mortality following infarction.

There has also been interest in the development of therapies capable of providing additional myocardial protection which could be administered in conjunction with thrombolytic therapy, or alone, because retrospective epidemiological studies have shown

that mortality during the first few years following infarction appears to be related to original infarct size.

Polyethylene glycol.

PEG (α-Hydro-ω-hydroxypoly-(oxy-1,2-ethanediyl)) is known by numerous designations including macrogol; PEG; Carbowax; Jeffox; Nycoline; Pluracol E; Poly-G; Polyglycol E; and Solbase. PEG refers to liquid and solid polymers containing one or more sub-units of the general formula $-(OCH_2-CH_2)_n-$, where *n* is greater than or equal to 4. In general, each PEG is followed by a number that corresponds to its average MW. PEG syntheses are described in Hibbert (1939) *J. Am. Chem. Soc.* 61:1905-1910. For review see Powell, III in *Handbook of Water-Soluble Gums & Resins*, R.L. Davidson ed. (McGraw-Hill, New York, 1980) pp. 18/1-18/31.

PEGs are used as water-soluble lubricants for rubber molds, textile fibers, and metal-forming operations, in food and food packaging, in hair preparations and in cosmetics in general and as ointment and suppository bases in pharmaceutical compositions.

Typically, PEGs are clear, viscous liquids or white solids that dissolve in water to form transparent solutions. They are soluble in many organic solvents and readily soluble in aromatic hydrocarbons. They are only slightly soluble in aliphatic hydrocarbons. Typically, they do not hydrolyze on storage. PEGs have low toxicity. The molecular weights of PEG compositions listed herein are given in number averages rather than weight averages.

PEG20M consists of two or more molecules of PEG having approximate molecular weights of 6000-10,000 joined by a bisphenol epoxide linker (CAS # 37225-26-6; CAS name Oxirane, 2, 2'-[(1-methylethylidene)bis(4,1-phenyleneoxymethylene)]bis-, polymer with (α-Hydro-ω-hydroxypoly(oxy-1,2-ethanediyl). PEG20L is a substantially linear PEG having an average molecular weight of about 20,000 Daltons (available from several commercial sources including, but not limited to, Shearwater Polymers (Huntsville, AL), Clariant/Hoechst Celanese (Muttenz, Switzerland), Fluka (Buchs, Switzerland) and Nippon Oils and Fats (Tokyo, Japan)). Various other molecular weights of linear PEG are also available from several commercial sources.

More recently, PEG has been used in a number of pharmacological applications. PEGs are frequently chosen for carriers in pharmacological applications because they are

generally viewed as inert additives. For example, PEGs are often conjugated to oligonucleotides for in vivo delivery because the PEG acts "as a soluble, inert synthetic support." Bonora, G.M., et al., "Synthesis and Characterization of High-Molecular Mass Polyethylene Glycol-Conjugated Oligonucleotides," Bioconjug. Chem. 8:793-7 (1997). The conjugation of PEG to foreign proteins, such as cytokines and antibodies, reduces the immune response triggered when the proteins are administered into test mammals. U.S. Patent Nos. 5,447,722; 4,902,502; 5,089,261; 5,595,732; 5,559,213; and 4,732,863. Conjugation to PEG also increases the solubility and biological half-life of cytokines. WO 8700056 and U.S. Patent No. 5,089,261. Conjugates of PEG and glucocerebroside have been formulated for treating Gaucher's disease. WO 9413311. PEG has also been conjugated to such enzymes as adenosine deaminase, amidase bovine and asparaginase, for therapeutic use. *See* Delgado et al. (1992) *Crit. Rev. Ther. Drug. Carrier Syst.* 9:249-304; and Burnham (1994) *Am. J. Hosp. Pharm.* 51:210-218, for review.

Organ preservation.

Transplantation of vital organs such as the heart, liver, kidney, pancreas, and lung has become increasingly successful and sophisticated in recent years. Because mammalian organs progressively lose their ability to function during storage, even at freezing temperatures, transplant operations need to be performed expeditiously after organ procurement so as to minimize the period of time that the organ is without supportive blood flow. This reduces the availability of organs to patients in need of transplants.

In clinical practice, the two major situations in which cardiac preservation is required are heart transplantation and during and after cardioplegia for open-heart surgery. In heart transplantation, the donor heart is exposed through a midline sternotomy. After opening the pericardium, the superior and inferior vena cavae and the ascending aorta are isolated. The venous inflow is then occluded, the aorta is cross clamped, and approximately 1 liter of cold organ preservation solution (OPS) is flushed into the aortic root under pressure through a needle; as a result, the heart is immediately arrested. Cooling is supplemented by surrounding the heart with iced saline. The chilled, arrested heart is then surgically excised, immersed in cold OPS, packed in ice and rushed to the recipient center.

The recipient's chest is opened through a midline sternotomy, and after placing the patient on cardiopulmonary bypass, the diseased heart is excised. The preserved donor

heart is then removed from the OPS, trimmed appropriately and sewn to the stumps of the great vessels and the two atria in the recipient chest. After completion of the vascular anastomoses, blood is allowed to return to the heart. The transplanted heart will then either resume beating spontaneously or will require chemical and electrical treatment to restore normal rhythm. When the heart is ready to take over the circulation, the cardiopulmonary bypass is discontinued and the recipient's chest closed.

Most non-transplant surgical procedures on the heart, such as coronary artery bypass grafting, require that the heart's action be arrested for a period ranging from 1 to 4 hours. During this time, the heart is kept cool by external cooling as well as by periodically reflushing an OPS through the coronary arteries. The OPS composition is designed to rapidly arrest the heart and to keep it in good condition during the period of standstill so that it will resume normal function when the procedure is finished.

In cardioplegic procedures, the heart is exposed in the chest and, at a minimum, the aortic root is isolated. A vascular clamp is applied across the aorta and approximately 1 liter of cold OPS, which can, but does not always, contain some patient blood (referred to as "blood cardioplegia") is flushed into the aortic root through a needle. Venting is provided through the left ventricle, pulmonary artery or the right atrium and the effluent, which can contain high levels of potassium, is sucked out of the chest. This, together with external cooling, produces rapid cessation of contractions. During the period of arrest, the patient's circulation is maintained artificially using cardiopulmonary bypass.

After completion of the surgical procedure, blood flow is restored to the coronary circulation and heartbeat returns either spontaneously or after chemical and electric treatment. The ease with which stable function is restored depends to a large extent on the effectiveness of preservation by the OPS. Once the heart is beating satisfactorily, cardiopulmonary bypass is discontinued and the chest closed. General methods for organ transplant and heart surgery are disclosed in D.K.C. Cooper (editor), *The Transplantation and Replacement of Thoracic Organs*, Boston, Kluwer Academic Publishers (1997); and Collins et al. (1992) *Kidney International* 42:S-197-S-202 and the art cited therein, and are commonly known in the art.

It is generally understood that "living" organs, including the heart, continue the process of metabolism after removal from the donor so that cell constituents are continuously metabolized to waste products. If the storage technique is inadequate, the accumulation of these metabolic waste products, depletion of cell nutrients and consequent

derangement of cell composition lead to progressive loss of function and ultimately to cell death. That is, the organ will lose its ability to function adequately after transplantation into the recipient. Several procedures have been explored to successfully enable organ preservation *ex vivo* for useful time periods. In one method, the donor organ is cooled rapidly by flushing cold solutions through the organ's vascular system and is maintained at temperatures near 0°C for the purpose of greatly slowing the metabolic rate. In the case of the mammalian heart, the flush solution composition is designed to cause the heart to rapidly stop beating as well as to preserve it.

In 1988, University of Wisconsin (UW) solution was introduced. Belzer et al. (1988) *Transplantation* 45:673-676. This solution, capable of preserving the pancreas and kidney for 72 hours, and the liver for 30 hours, subsequently became the standard organ preservation solution for transplant surgery and the benchmark against which other OPS compositions were measured. However, the heart is more recalcitrant to long-term storage than other organs, and UW solution is unreliable for storage of hearts for as short a period as 24 hours. Wicomb et al. (1989) *Transplantation* 47:733-734.

Improvements in the design of OPS compositions, as reviewed in Collins et al. (1992) *Kidney International* 42:S-197-S-202 and others described in the art, have proceeded along several paths, including: (1) modification and simplification of UW solution; (2) investigation of organ-specific requirements; (3) addition of pharmacological agents, particularly calcium antagonists for control of acidosis; (4) the use of a terminal rinse solution; and (5) the use of solutions containing PEG.

Wicomb et al. reported the beneficial effects of PEG 8000 on rabbit hearts preserved by oxygenated low-pressure perfusion for 24 hours; this solution also contained horseradish peroxidase. Wicomb et al. (1989) *Transplantation Proceedings* 21:1366-1368. The substitution of PEG20M for hydroxyethyl starch (HES) as the colloid in UW solution also yielded excellent cardiac function. The substitution of PEG20M for HES also allowed baboon heart storage up to 48 hours and increased cardiac output (CO) under conditions of microperfusion. Wicomb et al. (1986) *J. Surg. Res.* 40:276; and Wicomb et al. (1989) *Transplantation* 48:6-9. "Microperfusion" is a hypoxic, very-low-flow perfusion with flow rates such as 3 mL/g heart wt/24 hour, which is 1/500 of that typical of conventional continuous perfusion. Wicomb et al. (1989) *Transplantation* 48:6-9.

An improved OPS, Cardiosol™ heart preservation solution, contained the substitution of PEG20M for HES and eliminated five components of UW solution

(penicillin, dexamethasone, insulin, allopurinol, and adenosine). Wicomb et al. (1990) *Transplantation* 49:261-264; and United States Patent No. 4,938,961. Cardiosol™ heart preservation solution contains 5% or 10% by weight PEG 20M (Union Carbide Chemicals and Plastics Co., Inc., Charleston, WV), 40 mM sodium, 125 mM potassium, 5 mM magnesium, 25 mM phosphate, 5 mM sulfate, 100 mM lactobionate, 30 mM raffinose, and 3 mM glutathione. Collins et al., *The Lancet* 338:890-891 (1991); and Wicomb et al. (1994) *J. Heart Lung Transplantation* 13:891-894. This solution was found to be superior to UW solution both for 4-hour hypothermic and 24-hour microperfusion storage. Collins et al. (1992).

The mechanism of PEG activity in OPS in unknown. However, as reviewed in Collins et al. (1992), PEG is known to improve tissue viability, reduce ischemic injury by preventing cell swelling, interact with lipids in the cell membrane. OPS containing PEG20M also appears to blunt the immune response to a transplanted organ previously flushed with a PEG solution. Itasaka et al. (1994) *Transplantation* 57:645-648; and Tokunaga et al. (1992) *Transplantation* 54:756-758. PEG20M has also been proposed to be effective when used only as a terminal rinse solution after a period of ice storage. Collins et al. (1992).

The drawback of using PEG20M in OPS solution is its vulnerability to oxidative damage. Oxidative damage results in changes to the PEG20M structure and concomitant problems with instability of the OPS solution during storage and use. Thus, there is a need for an improved composition for use in OPS solutions and other aspects of preserving or restoring the function of cells, tissues or organs.

Gastrointestinal Disorders.

A variety of food supplements containing, in part, partially processed plant extracts have been used to ameliorate the gastrointestinal disorders that often accompany chemotherapy, radiation and AIDS. The supplements generally contain carbohydrates, fat and plant protein hydrolysates. See, e.g., Tomei and Cope *et al.* in *Apoptosis: The Molecular Basis of Cell Death* (1991) Cold Spring Harbor Laboratory Press. PCT Publication No. WO 95/15173 and U.S. Patent Nos. 5,620,885, 5,567,425, 5,635,186 and 5,624,672 describe plant-derived extracts that produce an antiapoptotic effect. It has now been found that these extracts contain the following phospholipids: 18:1-lysophosphatidic acid (18:1-LPA), lysophosphatidylcholine (LPC), lysophosphatidylinositol (LPI),

phosphatidic acid (PA) and phosphatidylinositol (PI) in the ratios of approximately 2:1:2:20:20, by weight in addition to various optional protein and carbohydrate constituents.

A method of preserving or restoring cell, tissue, or organ function, and/or preventing apoptosis would be useful for a variety of therapeutic uses, particularly organ preservation.

All references cited herein are incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The present invention encompasses methods of use of therapeutically effective compositions containing polyethylene glycol (PEG) which have been found to exhibit antiapoptotic activity or to protect, preserve or restore cell, tissue or organ function.

Accordingly, one aspect of the invention is a method of treating apoptosis, preserving or restoring function in cells, tissue or organs, comprising administering a therapeutically effective amount of a pharmaceutically acceptable composition comprising a PEG Based Molecule, wherein said pharmaceutically acceptable composition does not contain enough potassium to cause cardioplegia in a beating human heart. In a more specific embodiment of the present invention, the PEG Based Molecule has the formula R_1O-$(CH_2CH_2O)_n$-R_3, wherein 'n' is an integer between 1 and about 1000, wherein said PEG Based Molecule comprises at least 4 ethylene oxide monomers, and wherein R_1 and R_3 are independently selected from the group consisting of H, CH_3, unsubstituted or substituted, linear or branched alkyl.

In various specific embodiments of the present invention, R_1 is H and R_3 is selected from the group consisting of linear alkyl and branched alkyl, or wherein R_1 is H and R_3 is H, or wherein R_3 is linear alkyl, or wherein R_3 is branched alkyl, or wherein 'n' is an integer between about 200 and about 1000, or wherein the linear alkyl group is selected from the group consisting of $-CH_2CH_2CH_2CH_2Ar$, $-CH_2CH_2CH(Ar)R_4$ and $-CH_2CH(O(CH_2CH(CH_3)O)_nR_4)CH_3$, wherein R_4 is selected from the group consisting of H, CH_3, linear alkyl, branched alkyl, aryl and substituted aryl, or wherein the linear alkyl group is selected from the group consisting of $-CH_2C(O)OR_4$, $-CH_2C(O)NR_5R_6$, $-CH_2CH_2C(O)OR_4$, $-CH_2C(O)NR_5R_6$, and wherein R_4, R_5 and R_6 are independently selected from the group consisting of H, CH_3, linear alkyl, branched alkyl, aryl and substituted aryl, or wherein the branched alkyl group is selected from the group consisting

of -$CH(CH_2OR_4)CH_2OR_7$, -$CH_2CH(CH_2CH_2OR_4)_2$ and -$CH_2CH_2CH(CH_2OR_4)CH_2CH_2OR_4$, wherein R_4 and R_7 are independently selected from the group consisting of H, CH_3, linear alkyl, branched alkyl, aryl and substituted aryl.

In one embodiment, the polyethylene glycol is a Star PEG or an otherwise branched polyethylene glycol, or specifically, wherein the star-shaped polyethylene glycol has up to approximately 100 arms, or wherein the branched polyethylene glycol has 3, 4, 6 or 8 arms. In a specific embodiment, the polyethylene glycol is a polyethylene glycol-polypropylene glycol copolymer or another random or block copolymer of PEG.

In a specific embodiment, the pharmaceutically acceptable composition contains less than 125 μM potassium.

In one embodiment of the method a patient is treated with a PEG Based Molecule composition of the present invention, wherein the patient is suffering from a gastrointestinal perturbation which could be caused by a variety of stimuli. Human immunodeficiency virus, chemotherapeutic agents, radiation and infectious diseases are examples of such stimuli. In particular, examples of infectious diseases include inflammatory bowel disease and diseases selected from the group consisting of diarrhea-causing organisms. In addition, said treatment decreases immunodeficiencies associated with immunosuppressing viruses such as human immunodeficiency virus, chemotherapeutic agents, or radiation and immunosuppressive drugs mentioned above.

In another embodiment, the composition comprising PEG Based Molecule is administered to a patient who is undergoing or has recently undergone ischemia and/or reperfusion damage associated with a variety of conditions subsequent to ischemia. Coronary artery obstruction; cerebral stroke; spinal stroke; spinal/head trauma and concomitant severe paralysis and frostbite are examples of such conditions.

In another embodiment of the invention is a method of treating apoptosis, which involves topically administering the composition comprising PEG Based Molecule to a patient suffering from a variety of dermatological conditions. Wrinkling, sagging, psoriasis, baldness, wound healing and hair loss are examples of such conditions.

Also encompassed by the present invention is a method of treating apoptosis, preserving or restoring function in cells, tissue or organs, comprising administering a therapeutically effective amount of a pharmaceutically acceptable composition comprising a Branched PEG Based Molecule wherein said Branched PEG Based Molecule does not decompose to release a phenol based compound. In a specific embodiment, the Branched

PEG Based Molecule comprises between about 200 and about 1000 ethylene oxide monomers.

In another aspect of the invention, methods are provided for preserving or restoring function in cells, tissues or organs, comprising administering a therapeutically effective amount of a pharmaceutically acceptable aqueous solution, consisting essentially of a PEG Based Molecule.

In another aspect of the invention, methods include treatment of apoptosis by administration to a patient in need of such treatment, a therapeutically effective amount of a pharmaceutically acceptable agent containing a composition comprising a Linear PEG Based Molecule, wherein said Linear PEG Based molecule has a molecular weight greater than 8,000 Daltons.

Other methods using the claimed compositions include treating apoptosis *in vitro*. These methods comprise treating cells with a composition comprising a tissue culture media and an effective amount of A PEG Based Molecule. The cultured cells include, without limitation, eukaryotic and prokaryotic cells, such as bacterial cells, plant cells, yeast cells, fungi cells, insect cells, mammalian cells, and human cells in particular. In one embodiment, the cells are part of a tissue or organ.

The present invention encompasses a method of organ preservation comprising adding an effective amount of a composition comprising a PEG Based Molecule to the solution in which the organ is stored, wherein said solution does not contain enough potassium to cause cardioplegia in a beating human heart.

The present invention further encompasses a method of organ preservation comprising administering to the host animal at least one intravenous bolus of an effective amount of a composition comprising a PEG Based Molecule, wherein said composition does not contain enough potassium to cause cardioplegia in a beating human heart.

In an additional embodiment, the present invention encompasses a method of treating cardiac ischemia comprising administering a therapeutically effective amount of a pharmaceutically acceptable composition comprising a PEG Based Molecule to a human or animal in need of such treatment. In a specific embodiment, this treatment protects tissue from infarct damage and/or reduces the level of ST segment shift as measured by electrocardiogram. Specifically, the PEG Based Molecule can comprise a Linear or Branched PEG Based Molecule.

In another embodiment of the present invention, cardiac pathology associated with ST segment shift as measured by electrocardiogram is treated by administering a therapeutically effective amount of a pharmaceutically acceptable composition comprising a PEG Based Molecule to a human or animal in need of such treatment. In various specific embodiments, the PEG Based Molecule can comprise a Linear or Branched PEG Based Molecule, the composition further can comprise a phospholipid, and the phospholipid can be a lysophospholipid.

In another embodiment, the present invention encompasses a method of treatment comprising administering to a patient a therapeutically effective amount of a pharmaceutically acceptable composition comprising a polyethylene glycol and an LPA to a patient in need of such treatment. The LPA encompasses lysophosphatidic acid analogs as described herein. In a specific embodiment the patient is suffering from a condition related to apoptosis, ischemia, traumatic injury, or reperfusion damage. Alternatively, the treatment diminishes apoptosis-related problems associated with immunosuppressing viruses, chemotherapeutic agents, or radiation and immunosuppressive drugs. The virus can be human immunodeficiency virus. In a specific embodiment, the patient is undergoing ischemia and/or reperfusion subsequent to ischemia. More specifically, the present invention encompasses a method whereby the reperfusion is associated with coronary artery obstruction; cerebral or spinal stroke; spinal/head trauma and concomitant severe paralysis; frostbite; coronary angioplasty; tissue attachment, such as, without limitation, small bowel tissue attachment, blood vessel attachment; limb attachment; organ attachment; bypass surgery; and kidney reperfusion.

In yet another embodiment, an organ transplant recipient is treated by administering to the recipient an amount of a composition comprising a PEG Based Molecule effective to enhance function of the transplanted organ.

Generally, a method of treating a patient comprising administering to the patient a therapeutically effective amount of a composition comprising a PEG Based Molecule is encompassed by the invention.

A specific method is encompassed for preventing or reducing the severity of myocardial infarction which comprises administering to a subject of a coronary occlusion, after the occurrence of the coronary occlusion and prior to the onset of a myocardial infarction, a myocardial-infarction-preventing amount of a compound comprising a PEG Based Molecule. This composition can also contain a lysophospholipid.

In another embodiment of the invention is a method of treating apoptosis that involves topically administering the composition to a patient suffering from a variety of dermatological conditions. Wrinkling, sagging, psoriasis, baldness and hair loss are examples of such conditions.

In another aspect of the invention, methods are provided for making appropriate compositions for practice of the present invention. One method includes adding to a physiologically acceptable buffer an amount of a PEG Based Molecule effective to produce antiapoptotic activity. Another method includes adding a phospholipid, specifically a lysophospholipid.

Other methods using the claimed compositions include treating apoptosis *in vitro*. These methods comprise treating cultured cells with a composition comprising a tissue culture media and an effective amount of a PEG Based Molecule. The cultured cells include, without limitation, eukaryotic and prokaryotic cells, such as bacterial cells, plant cells, yeast cells, fungi cells, insect cells, mammalian cells, and human cells in particular. In one embodiment, the cells are part of a tissue or organ.

The present invention encompasses method of organ preservation comprising adding an effective amount of a composition comprising a PEG Based Molecule to the solution in which the organ is stored.

The present invention further encompasses a method of organ preservation comprising administering to the host animal at least one intravenous bolus of an effective amount of a composition comprising a Linear PEG Based Molecule.

In an additional embodiment, the present invention encompasses a method of treating cardiac ischemia comprising administering a therapeutically effective amount of a pharmaceutically acceptable composition comprising a PEG Based Molecule to a human or animal in need of such treatment. In a specific embodiment, this treatment protects tissue from infarct damage and/or reduces the level of ST segment shift as measured by electrocardiogram. Specifically, the PEG Based Molecule can comprise PEG20L.

In another embodiment of the present invention, cardiac pathology associated with ST segment shift as measured by electrocardiogram is treated by administering a therapeutically effective amount of a pharmaceutically acceptable composition comprising a PEG Based Molecule to a human or animal in need of such treatment. In various specific embodiments, the PEG Based Molecule can comprise PEG20L, the composition

further can comprise a phospholipid, and the phospholipid can be lysophosphatidic acid or an analog thereof.

In another embodiment, the present invention encompasses a method of treatment of a patient comprising administering to the patient a therapeutically effective amount of a pharmaceutically acceptable composition comprising a polyethylene glycol to a patient in need of such treatment. In a specific embodiment the patient is suffering from a condition related to apoptosis, ischemia, traumatic injury, or reperfusion damage. Alternatively, the treatment diminishes apoptosis-related problems associated with immunosuppressing viruses, chemotherapeutic agents, or radiation and immunosuppressive drugs. The virus can be human immunodeficiency virus. In a specific embodiment, the patient is undergoing ischemia and/or reperfusion subsequent to ischemia. More specifically, the present invention encompasses a method whereby the reperfusion is associated with coronary artery obstruction; cerebral or spinal stroke; spinal/head trauma and concomitant severe paralysis; frostbite; coronary angioplasty; blood vessel attachment; tissue attachment, such as without limitation small bowel attachment; limb attachment; organ attachment; and kidney reperfusion.

The present invention additionally encompasses both compositions for the treatment of myocardial infarction containing therapeutically effective quantities of a PEG Based Molecule and LPA, and methods of treating myocardial infarction by administering therapeutically effective quantities of a PEG Based Molecule and LPA.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph depicting the effect of PEG concentration and molecular weight on protection of C3H/10T½ cells from serum deprivation over 24 and 48 hours. PEG of molecular weights 8,000, 20,000 and 35,000 were used at concentrations of 1, 2 and 4 mM. The open section of the bars represents adherent, viable cells and the solid section of the bars represents non-adherent, dead cells. In this and all figures, where not specified as PEG20M or a branched PEG Based Molecule (such as 3-arm PEG) all PEGs are linear PEGs.

Figure 2 is a bar graph depicting the effect of PEG concentration, branching and molecular weight on protection of C3H/10T½ cells from serum deprivation over 24 hours. PEG of molecular weights 10,000 and 20,000 were used at concentrations of 0.5%, 2%, 5%, and 10% (weight per volume). The PEGs were either linear, 3 arm or 8 arm. The

open section of the bars represents adherent, viable cells and the solid section of the bars represents non-adherent, dead cells.

Figure 3 is a bar graph depicting the effect of polyethylene glycol (PEG) molecular weight on protection of C3H/10T½ cells from serum deprivation at 1 mM in the presence and absence of 1.5 μM 18:1-LPA. PEG of molecular weights 200, 1,000, 3,000, 6,000, 8,000, 20,000 and 35,000 were used. The open section of the bars represents adherent cells and the solid section of the bars represents non-adherent cells.

Figure 4 is a graph depicting the prevention of cardiomyocyte death induced by serum/glucose deprivation by PEG, Five Phospholipid Mixture (referred to in the figure as "5PM", and represented as μM of 18:1-LPA) and a mixture of PEG and Five Phospholipid Mixture. The squares represent no PEG, the circles represent 0.3% PEG and the triangles represent 2% PEG.

Figure 5 is a graph depicting the prevention of cardiomyocyte death induced by serum/glucose deprivation by PEG20L at different concentrations.

Figure 6 is a bar graph depicting the prevention of cardiomyocyte death induced by serum/glucose deprivation by 1 mM PEG of different molecular weights relative to cells maintained in complete serum.

Figures 7A - 7M are photomicrographs showing the prevention of ceramide-induced cardiomyocyte death by Five Phospholipid Mixture and mixtures of Five Phospholipid Mixture (referred to in the figures as "5PM") and PEG. Pictures A-F contained 10 μM ceramide and 0.3% PEG (B), 2% PEG (C), 5 μM 18:1-LPA presented as Five Phospholipid Mixture (D), 5 μM 18:1-LPA presented as Five Phospholipid Mixture plus 0.4% PEG (E), and 5 μM 18:1-LPA presented as Five Phospholipid Mixture plus 2% PEG (F). Picture G contained only serum and glucose and no active ingredients. Pictures H-M contain 15 μM ceramide and 0.3% PEG (I), 2% PEG (J), 5 μM 18:1-LPA presented as Five Phospholipid Mixture (K), 5 μM 18:1-LPA presented as Five Phospholipid Mixture, plus 0.4% PEG (L), and 5 μM 18:1-LPA presented as Five Phospholipid Mixture plus 2% PEG (M).

Figure 8 is a bar graph depicting the effect of the actual infarct size compared to the area at risk in the pig heart model treated with control (solid bar), PEG 20L (striped bar) and a dose of Five Phospholipid Mixture and PEG (gray bar - labeled "PEG + 5PM").

Figure 9 is a bar graph depicting the actual infarct size compared to the area at risk in the dog heart model treated with placebo (solid bar), a mixture of superoxide dismutase

(SOD) and catalase (striped bar) and a dose of the combination of Five Phospholipid Mixture ("5PM") (12.5 μg/kg) and PEG20L (25 mg/kg) (white bar).

Figure 10 is a graph depicting the addition of PEG plus Five Phospholipid Mixture (closed circles) and PEG plus 18:1-LPA (open circles) after ischemia preventing cardiomyocyte death induced by ischemia and reperfusion. The amount of 18:1-LPA corresponds to its quantity in the indicated amount of Five Phospholipid Mixture.

MODES FOR CARRYING OUT THE INVENTION

Polyethylene glycol (PEG) has generally served in the capacity of an inert carrier or transporter of therapeutic agents. The present invention provides compositions containing PEG and methods of using these compositions wherein the PEG component exhibits antiapoptotic activity and therapeutic effect, rather than simply acting as a carrier to deliver another active agent. Additionally, PEG acts to enhance the therapeutic effect of other antiapoptotic compounds.

In the process of evaluating other antiapoptotic compounds, their activities were tested with various carriers, of which one was PEG. Surprisingly, in the experiments using PEG as a carrier, the control samples containing only PEG showed antiapoptotic activity. Thus, various compositions comprising PEG were evaluated alone and found to display antiapoptotic activity in the absence of the phospholipids.

I. COMPOUNDS OF THE PRESENT INVENTION

The present invention relates to compositions comprising PEG and mixtures of compositions comprising PEG with other compounds.

A. Structure of compounds of the present invention.

1. Polyethylene glycol.

PEGs constitute a diverse group of molecules. Only compounds comprising one or more PEGs, wherein the compositions exhibit therapeutic efficacy and/or potentiate the therapeutic efficacy of LPAs, are suitable for use herein. It is within the skill of one skilled in the art to determine whether a particular composition comprising PEG is suitable for use in the claimed compositions. Such a determination can be made by any method known in the art, including the methods described herein.

A "PEG Based Molecule" is herein defined as a molecule comprising at least one "PEG unit", defined as a sub-unit of an ethylene oxide monomer having the general formula:

$$-(CH_2CH_2O)_n-$$

wherein n is greater than or equal to 4, or, wherein n is greater than or equal to one, and wherein the ethylene oxide monomer is repeated in the molecule at least 4 times.

In a more specific embodiment, PEG Based Molecules encompass a family of compounds that include, without limitation, compounds of the following formula or compounds containing one or more units of the following formula:

$$R_1O-(CH_2CH_2O)_n-R_3$$

where R_1 and R_3 are independently H, CH_3, or a linear or branched, substituted or unsubstituted alkyl group; and n is an integer between 1 and about 1,000 wherein at least 4 ethylene oxide monomers are present in the compound. PEG Based Molecules include molecules having both unsubstituted (R_1 and R_3 = H) as well as substituted PEG. Included, without limitation, in the term "compositions comprising PEG" are compositions where multiple PEGs of this general formula are joined by a central linking moiety into branched structures, such as, without limitation, $-CH_x(O-(CH_2CH_2O)_n-R_3)_v$, where x is 0, 1 or 2 and v is, respectively, 4, 3 or 2, and pentaerythritol, ethanolamine benzene and inositol based compounds, wherein one or more hydroxyl groups is substituted with $-O-(CH_2CH_2O)_n-R_3$.

Where R_1 and/or R_3 are linear, unsubstituted alkyl groups, such groups include, without limitation, $-CH_2CH_3$, $-CH_2CH_2CH_3$, $-CH_2CHCH_2$ and $-CH_2CCH$. Where R_1 and/or R_3 are linear, substituted alkyl groups, such groups include, without limitation - CH_2CO_2H, $-CH_2CO_2R_4$, $-CH_2C(O)NR_5R_6$, $-CH_2CH_2CO_2H$, $-CH_2CH_2CO_2R_4$, - $CH_2CH_2C(O)NR_5R_6$, $-CH_2CH_2C(O)H$, $-CH_2CH_2CH_2OH$, $-CH_2CH_2CH_2OR_4$, - $CH_2CH_2CH_2NR_5R_6$, $-CH_2CHOCH_2$, $-CH_2CH_2NHC(O)OR_4$, $-CH_2CH_2NHC(O)NR_5R_6$, - CH_2CH_2Ar, $-CH_2CH_2CH_2CH_2Ar$, $-CH_2CH_2CH(Ar)R_4$, $-CH_2CH(OH)CH_2OR_4$, - $CH_2CH(OH)CH_2NR_5R_6$, $-CH_2CH(O(CH_2CH_2O)_nR_3)-CH_2CH_2C(O)OR_4$, $-CH_2CH(O(CH_2CH_2O)_nR_3)CH_2CH_2C(O)OR_4$ and - $CH_2CH(O(CH_2CH(CH_3)O)_nR_4)CH_3$. The substituents, R_4, R_5 and R_6, of the linear, substituted alkyl groups are, independently, H, H_3C, linear or branched, substituted or

unsubstituted alkyl, aryl or substituted aryl; 'Ar' is an unsubstituted or substituted aryl group; 'n' is an integer between 1 and about 1000.

Where R_1 and/or R_3 are linear, substituted alkyl groups, one preferred alkyl group is $-CH_2CH_2CH(Ar)R_4$. Multibranch, star-shaped PEG (Star PEG) compounds contain such an alkyl group. Gnanou et al. (1988) Makromol. Chem. 189:2885-2892. The Star PEGs comprise between about 20 and about 200 individual polyethylene oxide units (arms) that are connected through substituents on the aryl group, which is at the PEG terminus. Preferably, the Star PEGs contain about 100 arms.

Another preferred linear, substituted alkyl group is $-CH_2CH(O(CH_2CH(CH_3)O)_nR_4)CH_3$. That alkyl group is contained within polyethylene oxide-polypropylene oxide copolymers. Chen et al. (1995) Proc. Intern. Symp. Contr. Rel. Bioact. Mater. 22:167. Such copolymers consist of 2 to about 100 alternating units of polyethylene oxide and polypropylene oxide. Additionally encompassed are other co-polymers, or block co-polymers of two or more different monomers where one of the monomers is a PEG unit.

Where R_1 and/or R_3 are branched, unsubstituted alkyl groups, such groups include, without limitation, $-CH_2CH(CH_3)_2$, $-CH_2CH(CH_3)CH_2CH_3$ and $-CH_2CH_2CH(CH_3)_2$. Where R_1 and R_3 are branched, substituted alkyl groups, such groups include, without limitation, $-CH(CH_2OR_4)CH_2OR_7$, $-CH_2CH(CH_2CH_2OR_4)_2$, $-CH_2CH_2CH(CH_2OR_4)-CH_2CH_2OR_4$. The substituents, R_4 and R_7, of the branched, unsubstituted alkyl groups are, independently, H, H_3C, linear or branched, substituted or unsubstituted alkyl, aryl or substituted aryl.

Where R_1 and/or R_3 are branched, substituted alkyl groups, a preferred alkyl group is $-CH(CH_2OR_4)CH_2OR_5$. Branched PEGs include, without limitation, compounds where R_4 is a polyethylene oxide unit, or a branched, alkyl polyethylene oxide unit, and R_5 is a branched, alkyl polyethylene oxide unit. The branched PEGs consist of at least 3 polyethylene oxide arms, and can also have 4 or more arms.

The PEGs of the present invention are either purchased from chemical suppliers or synthesized using techniques known to those of skill in the art. PEG20L is available from several commercial sources, including, but not limited to, Clariant (Germany), Fluka (Germany) and Nippon Oils and Fats (Tokyo, Japan). Several substituted PEGs are available from Shearwater Polymers, Inc. (Huntsville, Alabama), including the following: multibranched star-shaped PEGs (Star PEGs); branched PEGs (3, 4 or 8 arms); allyl PEG

($-CH_2CHCH_2$); PEG-aldehyde ($-CH_2CH_2CHO$); carboxymethylated PEG ($-CH_2C(O)OH$); PEG-propionic acid ($-CH_2CH_2C(O)OH$); and PEG-glycidyl ether ($-CH_2CHOCH_2$). Block polyethylene oxide-polypropylene oxide copolymers are available from BASF Corp. (Wyandotte, USA) under the tradename Pluronic®.

PEGs that contain an ester, amide, carbonate or carbamate substituted alkyl group are typically synthesized from an activated carbonyl compound. The nucleophilic addition of an alcohol (HOR_4) or an amine (HNR_5R_6) to the succinimidyl ester of carboxymethylated PEG (Shearwater Polymers, Inc.) provides $-CH_2C(O)OR_4$ and $-CH_2C(O)NR_5R_6$ substituted PEGs respectively. Royer and Anantharmaiah (1979) J. Am. Chem. Soc. 101:3394. The PEG substituents $-CH_2CH_2C(O)OR_4$ and $-CH_2CH_2C(O)NR_5R_6$ are produced upon the addition of an alcohol or amine to PEG succinimidyl propionate (Shearwater Polymers, Inc.). Royer and Anantharmaiah (1979). Carbonates and carbamates, $-CH_2CH_2NHC(O)OR_4$ and $-CH_2CH_2NHC(O)NR_5R_6$, are the reaction products of PEG-isocyanate (Shearwater Polymers, Inc.) and an alcohol or amine. Greenwald et al. (1995) J. Org. Chem. 60:331-336.

Other substituted PEGs are made using a variety of methods. Star PEGs and branched PEGs, when not purchased, are synthesized using polymerization or oligomerization reactions. Gnanou et al. (1988); and Rein et al. (1993) Acta Polymer 44:225. The nucleophilic addition of an alcohol or amine to the epoxide unit of PEG-glycidyl ether provides $-CH_2CH(OH)CH_2OR_4$ and $-CH_2CH(OH)CH_2NR_5R_6$ substituted PEGs. Pitha et al. (1979) Eur. J. Biochem. 94:11; and Elling and Kula (1991) Biotech. Appl. Biochem. 13:354. PEGs containing the linear alkyl group $-CH_2CH_2CH_2NR_5R_6$ are produced upon the reductive amination of PEG-aldehyde with HNR_5R_6. Chamow et al. (1994) Bioconjugate Chem. 5:133; and Wirth et al. (1991) Bioorg. Chem. 19:133.

As described herein, it has now been found that organ preservation solutions containing PEG Based Molecules without bisphenol epoxide linkers (-phenol-$C(CH_3)_2$-phenol-) exhibit improved therapeutic activity over those preservation solutions containing PEG20M, and further that organ preservation solutions containing PEG potentiate the antiapoptotic activity of LPAs and phospholipid mixtures. The invention thus encompasses therapeutically effective amounts of compositions comprising PEG or such compositions combined with one or more LPAs. The invention further encompasses compositions comprising PEG in amounts effective to potentiate the antiapoptotic activity of one or more LPAs, and mixtures thereof.

Preferably, the PEG is present in amounts effective to create therapeutic effect on its own or to potentiate the antiapoptotic activity of one or more LPAs as an antiapoptotic agent. PEG can be in the molecular weight range from about 1,000 to about 500,000 or higher. Preferably, the PEG has an average molecular weight of about 8,000 to about 100,000. More preferably, the PEG has an average molecular weight of from about 20,000 to about 40,000. Most preferably, the PEG has an average molecular weight of about 20,000.

Preferably, the compositions containing PEG do not degrade to release a compound containing a phenol moiety (a "phenol based compound"). Compositions comprising PEG that do not contain a bis-phenol-epoxide linker are preferred embodiments of the present invention. The compounds lacking bis-phenol-epoxide linkers appear to be more stable against oxidative damage in several formulations than PEG20M. PEG20M breaks down into two PEG molecules over time, releasing a phenol based compound in the process, which is believed to damage cells (Data not shown). Examples, below, show enhanced preservation is obtained by using PEG containing compositions that do not degrade to release phenol based compounds.

By "Linear PEG Based Molecule" is meant a PEG Based Molecule having a substantially linear primary structure. For example, branches do not emanate from the backbone of a Linear PEG Based Molecule. By "Branched PEG" is meant a PEG Based Molecule that is not a Linear PEG Based Molecule. PEG 20L is a Linear PEG Based Molecule. PEG20M is a non linear PEG Based Molecule.

Experience in our laboratory suggests that certain samples of PEG20L may not be suitable for use in the compositions described herein. Therefore, it is preferred to test each sample as described herein before use. Linear PEG Based Molecules of between about 20,000 and about 35,000 molecular weight is also preferred. Linear PEG Based Molecules of higher molecular weight may have clearance problems when administered in vivo. Thus, Linear PEG Based Molecules of molecular weight greater than 35,000 are preferably used topically.

Also preferred for some methods of the present invention are compositions comprising two or more distinct units of PEG, particularly where the subunits are located on different branches of the molecule. Without limiting the invention to any particular mechanism of action, it is believed that compositions containing more than one subunit of PEG, particularly where the subunits are located on different branches of the molecule,

display lower viscosity, which improves the clearance profile of the molecule *in vivo* and allows for the use of higher molecular weight PEG compositions without introducing undesirably high viscosity.

By "having an average molecular weight of about 20,000 Daltons" is meant that individual linear polymers can vary in length, but the average molecular weight is about 20,000 Daltons. Those of ordinary skill in the art will appreciate that synthetic polymers such as PEG cannot be prepared practically to have exact molecular weights, and that the term "molecular weight" as used herein refers to the average molecular weight of a number of molecules in any given sample, as commonly used in the art. For example, a sample of PEG 2,000 might contain a statistical mixture of polymer molecules ranging in weight from, for example, 1,500 to 2,500 Daltons with one molecule differing slightly from the next over a range. Specification of a range of molecular weight indicates that the average molecular weight can be any value between the limits specified, and can include molecules outside those limits. The molecular weight distribution of a PEG can be determined by size-exclusion chromatography (SEC), a technique known in the art, using, for example, a combination of columns to achieve resolution from 1,000,000 to 200 molecular weight. PEG standards from 100,000 to 1,400 molecular weight can be used for calibration.

The oxidation rate of various PEG containing compositions is dependent upon a combination of storage conditions including: (1) whether stored as a solid or in solution; (2) temperature; (3) exposure to light; and (4) the availability of oxygen. In one embodiment, the compositions of the present invention are protected from ambient light, and are stored at below room temperature, or specifically at about 2-8 °C.

By "anoxically" is meant the absence of oxygen, a condition that can be maintained by purging with argon or nitrogen gas, and then packaging in a gas-impervious container. The absence of oxygen need not be total. Preferably, it is below about 10% of the total gas present in the sample. More preferably, it is below about 1% of total gas present in the sample. In one embodiment of the present invention the composition comprising PEG is stored anoxically.

Preferably, the composition comprising PEG is present in an effective concentration and is essentially free of decomposition products.

By "essentially free" is meant that the levels of decomposition products in a composition comprising PEG do not completely negate the ability of the composition to inhibit apoptosis or to protect, preserve or restore cell, tissue or organ function.

By "decomposition products" is meant the products produced when PEG (linked or unlinked) is oxidized or otherwise decomposed. Ultrafiltration or dialysis can reduce the levels of these decomposition products. Where the PEG has not decomposed appreciably and does not contain amounts of impurities that effect its antiapoptotic or protective activity, ultrafiltration may not be necessary.

Ultrafiltration can be performed using a Regenerated Cellulose Ultrafiltration Membrane or a Polyethersulphone Ultrafiltration Membrane or using other methods known in the art; dialysis can be performed using a Cellulose Acetate Hollow Fiber Dialyzer (Baxter Model CA 110) or using other methods known in the art. The membrane preferably has a molecular weight cut off of approximately 7,000 - 20,000 Daltons to allow removal of contaminating materials, including, but not limited to: oxidation products, ionic materials, metals and other contaminants. When compositions comprising PEG are prepared on a large scale, ultrafiltration is preferable to dialysis due to the relative speeds of processing large volumes. Preferably, ultrafiltration or dialysis consists of at least 1-2 changes of H_2O, or ultrafiltration or dialysis against at least a 1-2X volume of water. More preferably, ultrafiltration or dialysis consists of at least 3 changes of pure water, or ultrafiltration or dialysis against at least a 3X volume of water. Where a PEG contains high levels of decomposition products or other contaminants, it is preferred that ultrafiltration or dialysis is performed against sufficient volumes of water, or changes of water, to ensure that the contaminants are reduced to acceptable levels.

By "therapeutically effective amount" is meant a concentration of the component able to improve or ameliorate a condition related to apoptosis, any ischemic event, traumatic injury and reperfusion. Preferably, PEG is present in a therapeutically effective amount. In the case of OPS, an effective amount is the amount required to improve the ability of an OPS to preserve organs.

Screening steps are provided herein that guide one to determine whether a specific composition comprising PEG will be therapeutically or biologically effective. Guidance in selection can be obtained by analyzing the following parameters: (1) Aldehyde Levels, and (2) Cell Based Assay Performance.

(1) Aldehyde Levels

The following test is described generally by Gad Avigad, in "A Simple Spectrophotometric Determination of Formaldehyde and Other Aldehydes: Application to

Periodate-Oxidized Glycol Systems," Analytical Biochemistry 134:499 (1983), and has been modified as follows:

A solution of PEG for testing is prepared by dissolving 1 g PEG in 10 mL deionized water. 100 μL and 200 μL aliquots of this solution are transferred to borosilicate tubes. 100 μL of deionized water is then added to each 100 μL sample.

Calibration standards (200 μL each) consist of the following concentrations of a fresh formaldehyde solution: 0.0, 0.6, 1.9, 3.1, and 4.4 mg/L.

To each 200 μL solution of calibration standards and samples, prepared in triplicate in borosilicate tubes, 300 μL of a 1% 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (AHMT) solution in 1 M NaOH is added and vortexed to mix well. The reaction mixture is shaken at approximately 150 rpm on a rotary shaker at ambient temperature for 30 minutes. 500 μL of 0.2% sodium borohydride solution in 1 M NaOH is then added to stop each reaction, and vortexed to mix well. Each sample is transferred to a 1-mL polystyrene cuvette.

The spectrophotometer is calibrated and the absorbance of each standard/sample at 545 nm is measured to determine the approximate concentration of aldehyde present in the PEG tested. In a preferred embodiment, PEGs containing aldehyde levels above 17 mole-percent are not used in methods of the present invention.

(2) Cell Based Assay Performance

Several cell based assays can be used to evaluate the biological or therapeutic effectiveness of PEG containing compositions, including, but not limited to, a CH3/10T½ cell assay, described in the experimental section below, or an in vitro cardiomyocyte assay, as follows.

Cardiomyocytes from neonatal or infant rats are prepared by trypsinization and mechanical disaggregation and resuspended in Modified Eagles Medium (MEM), lxMEM vitamins (Gibco), 5% fetal bovine serum and 50 U/mL penicillin-G and pre-plated for 30 minutes to reduce contamination of non-myocytes. The non-adherent cardiac myocytes are separated and seeded in 35 mm dishes at a density of approximately 3.5×10^5 viable cells per mL. The cells are allowed to adhere for 16-24 hours in a 37°C/5% CO_2 humidified incubator.

Serum deprivation is performed by replacing the medium with fresh serum-free RPMI Media, whereas serum/glucose deprivation is performed using glucose-free RPMI. As a model of ischemia, cultures in serum and glucose free RPMI are placed in an airtight

chamber and the latter is continuously perfused with oxygen-free gas overlay of 95% N_2/5% CO_2 for 8 hours at 37°C. To model reperfusion of the ischemic cells, 10% fetal bovine serum, 2 g/L of glucose are added and the cultures are returned to a normal oxygen gas overlay (37°C/5% CO_2) in a humidified incubator for 16 hours.

To investigate the biological efficacy of a particular sample comprising PEG, the sample is added to cells in serum free medium at the beginning of cytotoxic treatment.

Because cardiomyocytes are terminally differentiated non-dividing cells, viability is then determined by measurement of the decrease in the relative number of adherent cells. Adherent cardiomyocytes are collected from culture dishes using 0.25% Trypsin/0.05% EDTA and counted on Coulter Counter ZM and Coulter Channelyzer 256.

In a preferred embodiment of the present invention, the compositions comprising PEG will display biological activity in that they reduce the level of cardiomyocyte cell death following serum and glucose deprivation when compared to similarly deprived control cells not treated with the compositions comprising PEG.

As with all primary cell culture assays, results of this assay may vary, and one skilled in the art will take such variability into account when interpreting the results of this assay.

2. Phospholipids

Compositions comprising PEG alone show significant apoptotic activity. These compositions also act synergistically with other agents, such as phospholipids, to achieve improved apoptotic activity.

Phospholipids are a class of phosphorus-containing lipids that are essential constituents of biological membranes. Various phospholipid preparations have been used for cooking, drug delivery (liposomes), slow release delivery systems, carrier media for hydrophobic drugs, gene transfer and replacement therapy, sunscreens, emulsions, anti-foaming agents, replacement of damaged or absent pulmonary surfactants, detergents and membrane stabilization. Phosphatidic acid (PA), phosphatidylinositol (PI), lysophosphatidic acids, lysophosphatidylinositol (LPI), and lysophosphatidylcholine (LPC) are found in a variety of plant and animal products. Lysophosphatidic acid analogs have been reported to have a variety of physiological activities including mitogenesis (i.e. prevention of hyperproliferative diseases), vasodilation, wound healing, anti-wrinkle and growth factor qualities. U.S. Patent Nos. 4,263,286; 4,746,652; 5,326,690; 5,480,877; 5,565,439; and 5,340,568. Lysophosphatidic acid is reviewed in detail by Moolenaar

(1994) *TICB* 4:213-219; Eichholtz et al. (1990) *Biochem. J.* 291:677-680; and Moolenaar (1995) *J. Biol. Chem.* 270:12949-12952.

While phospholipids have exhibited antiapoptotic effects, there are distinct difficulties encountered both in controlling the activity or bioavailability of the phospholipids and obtaining optimized balances of various phospholipids, particularly from plant extract methods. Thus, there is a need for a composition that can be combined with phospholipids to protect or enhance the activity of the phospholipids as well as assist in the manufacturing the antiapoptotic compositions.

In an additional aspect of the present invention, antiapoptotic activity is synergistically improved by the combination of LPA and PEG. In particular, PEG potentiates the antiapoptotic activity of LPA.

It was previously found that compositions containing certain ratios of five phospholipids are capable of producing an antiapoptotic effect as measured in *in vitro* cell assays designed to respond to an apoptosis signal. These compositions are readily obtainable from a variety of sources, including plants, animals and combinations of isolated or synthetic phospholipids. PCT WO 95/15173; and U.S. Patent No. 5,567,425. The phospholipids can be prepared synthetically, or semi-synthetically, by methods known in the lipid synthesis art. It has now been found that a variety of phospholipids display antiapoptotic activity in the absence of other phospholipids. The active ingredients of these antiapoptotic compositions were found to be in the combination of the phospholipids PA; PI; 18:1-LPA; LPI; and LPC.

The previously described phospholipids can be recombined to obtain reconstituted optimized mixture (ROM, or Five Phospholipid Mixture) which lacks the contaminants found in the native mixture. Five Phospholipid Mixture is described in PCT/US96/14752. Compositions comprising Five Phospholipid Mixture and PEG are an embodiment of the invention.

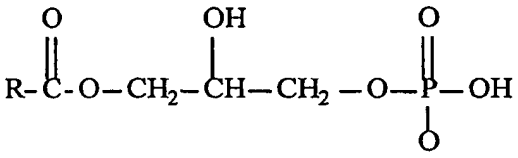
As defined herein, the compositions designated Five Phospholipid Mixture comprise the phospholipids, PA; PI; 18:1-LPA; LPI; LPC. The phospholipids are present in a range of ratios from 0–20:5–20:2–16:0–4:0–8, respectively. Preferably, these phospholipids are in a ratio of approximately 2-15:8-15:6-10:2-4:2-8, respectively. Most preferably, these phospholipids are in a ratio of approximately 10:10:8:2:4, respectively.

It has now been found that the major active component of Five Phospholipid Mixture is 18:1-LPA. The use of 18:1-LPA alone as a therapeutic has a number of well-

known drawbacks. 18:1-LPA alone has certain mitogenic activities, a short half-life both *in vivo* and under certain storage conditions. It has now been found that the antiapoptotic activity of 18:1-LPA and other LPAs, both alone and in combination with other phospholipids, are potentiated in the presence of PEG.

"LPA" is defined herein as a lysophosphatidic acid or analog thereof. Although phospholipid structures are well defined, they can vary with respect to lipid chain length and saturation. Typically, LPA has the following structures but can include other structures known in the art provided they are effective in producing therapeutic response.

LPA has the following general structure:



I

LPA is an acid in which only one of the hydroxyl groups of the glycerol is esterified to a fatty acid. Generically, LPA is a phosphatidic acid in which the sn-2 position of the glycerol moiety is not esterified and the sn-3 position is bound to the O-PO_3H_2 group, or, in the case of the salt, one or more hydrogen atoms are replaced, for example with Na^+. The sn-1 position contains an acyl ester of fatty acids. While natural LPAs occur with the phospho- moiety at the *sn*-3 position, synthetic LPAs can have alternative stereochemistry with, *e.g.*, the phospho- group at the *sn*-1 or *sn*-2 positions. The present invention encompasses these and other sterioisomers and positional isomers of LPA.

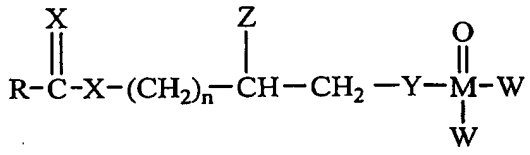
As used herein, LPA includes compounds having any one of a variety of fatty acids esterified at the #1 carbon position. Examples include compounds wherein the fatty acid ester is lauryl, myristyl, palmityl, stearyl, palmitoleyl, oleyl or linoleyl. For a representative example of suitable phospholipids see any chemical catalog of a phospholipid supplier, for instance, the (1994) Avanti Polar Lipids catalog, particularly pages 14 and 21.

R can be an unsubstituted or substituted, saturated or unsaturated, straight or branched chain alkyl having from 11 to about 24 carbon atoms. Substitutions include, but are not limited to, halogen, hydroxy, phenyl, amino or acylamino.

Given the examples provided herein, it can be determined readily if an LPA exerts sufficient antiapoptotic, or cell, tissue or organ preservation or restoration activity for the desired application. A wide variety of LPAs are known in the art and many of these can be purchased from commercial sources such as Avanti Polar Lipids Inc. (Alabaster, AL), or they can be synthesized by methods known in the art.

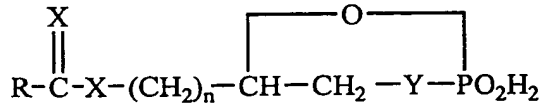
The term "UB" is used in reference to the various structures herein to describe the number of unsaturated carbon atoms in R. For example, if R is 18 and UB is 1, R contains 18 carbon atoms, with 1 unsaturated bond. Some LPAs are also referred to herein as R:UB-LPA (i.e. 18:1-LPA, wherein R is 18 carbon atoms with 1 unsaturated bond).

LPAs include, but are not limited to, the following structures:



II

or a cyclic phosphate derivative thereof having the structure:



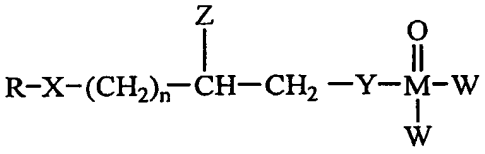
III

or pharmaceutically acceptable salts thereof, wherein each X is independently O or S; M is P or S, where when M is S, one W is (=O); each W is independently SH, OH, $OCH_2CH(NH_2)CO_2H$ and $OCHCH_3CH(NH_2)CO_2H$; each W is independently SH, OH, $OCH_2CH(NH_2)CO_2H$, $OCHCH_3CH(NH_2)CO_2H$, OPO_3H_2, or $OPO_2HOPO_3H_2$, where if one W is OPO_3H_2 or $OPO_2HOPO_3H_2$, the remaining W is OH; Z is OH, SH, NH_2, halogen, OPO_3H_2, H, $O(CH_2)_bCH_3$ where b=0 to about 2, or SO_3H; R is unsubstituted or substituted, saturated or unsaturated, straight or branched-chain alkyl having from preferably 5-7, more preferably 8-10 and most preferably, about 10 to preferably 24-30, more preferably 24-28 and most preferably about 24 carbon atoms, or $((CH_2)_mO)_p(CH_2)_mW$ where p is an integer from 1 to about 900 and where each m is independently an integer from 2 to about 10 and W is OH, or $O(CH_2)_qCH_3$ where q is an integer from 0 to about 10; Y is O or S; and n is an integer from 0 to about 10. Preferably,

R is between about 10 and 24, UB is 0-11, and mixtures thereof. More preferably, R is between about 14 and 20, UB is 0-6, and mixtures thereof. Even more preferably, R is between about 16 and 18, UB is 0-3, and mixtures thereof. Most preferably, R is 18, UB is 1 or 2, and mixtures thereof.

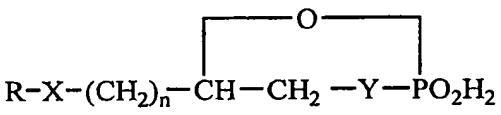
Methods of preparation of substitutions at the phosphate group of LPA have been described, and are included herein. Tokumura et al. (1981) *J. Pharm. Exp. Therap.* 219:219-224.

Also included in LPAs are ethers and thioethers at the C1 position having the structure:



IV

or a cyclic phosphate derivative thereof having the structure:



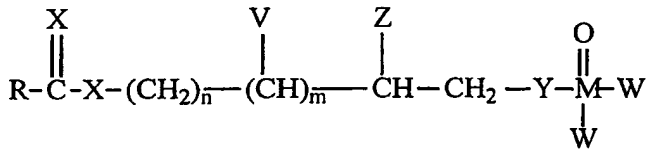
V

or pharmaceutically acceptable salts thereof, wherein X is O, S, or CH_2; M is P or S, where when M is S, one W is (=O); each W is independently SH, OH, $OCH_2CH(NH_2)CO_2H$ and $OCHCH_3CH(NH_2)CO_2H$; each W is independently SH, OH, $OCH_2CH(NH_2)CO_2H$, $OCHCH_3CH(NH_2)CO_2H$, OPO_3H_2, or $OPO_2HOPO_3H_2$, where if one W is OPO_3H_2 or $OPO_2HOPO_3H_2$, the remaining W is OH; Z is OH, CH_2OH, SH, NH_2, halogen, OPO_3H_2, H or SO_3H; R is unsubstituted or substituted, saturated or unsaturated, straight or branched-chain alkyl having from preferably 5-7, more preferably 8-10 and most preferably, about 10 to preferably 24-30, more preferably 24-28 and most preferably about 24 carbon atoms, or $((CH_2)_mO)_p(CH_2)_mW$ where p is an integer from 1 to about 900 and where each m is independently an integer from 2 to about 10 and W is OH, or $O(CH_2)_qCH_3$ where q is an integer from 0 to about 10; Y is O or S; and n is an integer from 0 to about 10. Simon et al. (1982) *Biochem. Biophys. Res. Comm.* 108:1743-1750.

Preferably, R is between about 10 and 24, UB is 0-11, and mixtures thereof. More preferably, R is between about 14 and 20, UB is 0-6, and mixtures thereof. Even more

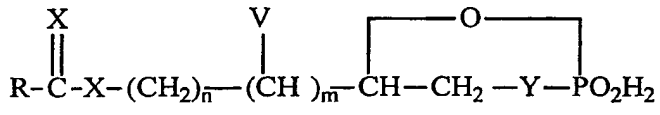
preferably, R is between about 16 and 18, UB is 0-3, and mixtures thereof. Most preferably, R is 18, UB is 1 or 2, and mixtures thereof.

Also included are glycero LPAs, having the structure:



VI

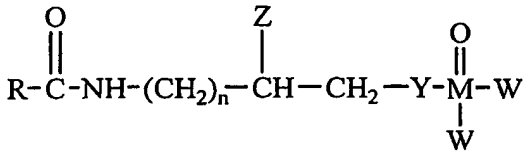
or a cyclic phosphate derivative thereof having the structure:



VII

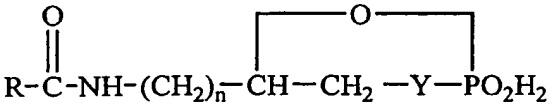
or pharmaceutically acceptable salts thereof, wherein each V is independently OH, SH, H, NH_2, halogen, OPO_3H_2, or OSO_3H; each X is independently O or S; M is P or S, where when M is S, one W is (=O); each W is independently SH, OH, $OCH_2CH(NH_2)CO_2H$ and $OCHCH_3CH(NH_2)CO_2H$; each W is independently SH, OH, $OCH_2CH(NH_2)CO_2H$, $OCHCH_3CH(NH_2)CO_2H$, OPO_3H_2, or $OPO_2HOPO_3H_2$, where if one W is OPO_3H_2 or $OPO_2HOPO_3H_2$, the remaining W is OH; Z is OH, SH, NH_2, halogen, OPO_3H_2, H or SO_3H; R is unsubstituted or substituted, saturated or unsaturated, straight or branched-chain alkyl having from preferably 5-7, more preferably 8-10 and most preferably, about 10 to preferably 24-30, more preferably 24-28 and most preferably about 23 carbon atoms, or $((CH_2)_pO)_q(CH_2)_pV$ where q is an integer from 1 to about 900 and where each p is independently an integer from 2 to about 10 and V is OH, or $O(CH_2)_bCH_3$ where b is an integer from 0 to about 10; Y is O or S; n is an integer from 0 to about 10; and m is an integer from 0 to about 10. Preferably, R is between about10 and 24, UB is 0-11, and mixtures thereof. More preferably, R is between about 14 and 20, UB is 0-6, and mixtures thereof. Even more preferably, R is between about 16 and 18, UB is 0-3, and mixtures thereof. Most preferably, R is 18, UB is 1 or 2, and mixtures thereof.

Also included are LPAs containing an amide bond and having the structure:



VIII

or a cyclic phosphate derivative thereof having the structure:



IX

or the reverse amide [structures VIII and IX, having R-**NH-C(=O)**-$(CH_2)_n$- in place of R-**C(=O)-NH**-$(CH_2)_n$-](NH-$CH_2)_n$-]or pharmaceutically acceptable salts thereof, wherein Z is OH, SH, NH_2, halogen, OPO_3H_2, H or SO_3H; M is P or S, where when M is S, one W is (=O); each W is independently SH, OH, $OCH_2CH(NH_2)CO_2H$ and $OCHCH_3CH(NH_2)CO_2H$; each W is independently SH, OH, $OCH_2CH(NH_2)CO_2H$, $OCHCH_3CH(NH_2)CO_2H$, OPO_3H_2, or $OPO_2HOPO_3H_2$, where if one W is OPO_3H_2 or $OPO_2HOPO_3H_2$, the remaining W is OH; R is an amino acid side chain or a branched amino acid side chain, or an alkylated amino acid side chain, unsubstituted or substituted, saturated or unsaturated, straight or branched-chain alkyl having from preferably 5-7, more preferably 8-10 and most preferably, about 10 to preferably 24-30, more preferably 24-28 and most preferably about 23 carbon atoms, or $((CH_2)_mO)_p(CH_2)_mW$ where p is an integer from 1 to about 900 and where each m is independently an integer from 2 to about 10 and W is OH, or $O(CH_2)_qCH_3$ where q is an integer from 0 to about 10; Y is O or S; and n is an integer from 0 to about 10. Preferably, R is between about 10 and 24, UB is 0-11, and mixtures thereof. More preferably, R is between about 14 and 20, UB is 0-6, and mixtures thereof. Even more preferably, R is between about 16 and 18, UB is 0-3, and mixtures thereof. Most preferably, R is 18, UB is 1 or 2, and mixtures thereof.

In all analogs containing W, it is preferred that W is not ethanolamine, glycerol, or choline.

In the above structures, where one W is SH, some molecules will exist as resonance structures, alternating between the (=O) and (=S) structures.

Naturally occurring derivatives are also encompassed in the term "LPA." Such derivatives include, but are not limited to, PHYPLA or cLPA. Murakami-Murofushi et al.

(1992) *J. Biol. Chem.* 267:21512-21517. Cyclic derivatives can also be synthesized by methods known in the art.

Pharmaceutically acceptable salts of the phospholipids encompassed by the present invention, include, but are not limited to, the free acid form, alkali metal salts, such as sodium and potassium; alkaline earth metal salts, such as calcium and magnesium; non-toxic heavy metal salts; ammonium salts; trialkylammonium salts, such as trimethylammonium and triethylammonium; and alkoxyammonium salts, such as triethanolammonium, tri(2-hydroxyethyl)ammonium, and tromethamine (tris(hydroxymethyl)aminomethane). Particularly preferred are sodium and ammonium salts.

The phospholipids can be obtained from any source including, but not limited to, commercial, isolated from a variety of different plants (including plant organs) and animals or created synthetically. Preferably the plants are in the soybean family, but the phospholipids can be isolated from other plants including, but not limited to, those in the *leguminosae* (beans and peas, etc.). The phospholipids can also be isolated from partially purified plant extracts including, but not limited to, soy molasses, lecithin, partially purified protein concentrates, partially purified protein hydrolysates and other soy fractions from which lipid can be extracted. It is within the skill of one in the art, utilizing the methods described herein, to determine whether the phospholipids of the present invention can be isolated from a particular species of plant, plant extract or organ within a plant. In addition, U.S. Patent No. 3,365,440 describes extraction of lipids from soybeans.

The phospholipids can be obtained from the plant sources by any method known in the art provided it results in purification of at least one of the phospholipids of the invention. A variety of methods are known in the art for purifying and analyzing phospholipids from plant sources. For review, see Bligh and Dyer (1959) *Can. J. Biochem. Physiol.* 37:911-917; Patton et al. (1982) *J. Lipid Res.* 23:190-196; Jungalwala (1985) Recent Developments in Techniques for Phospholipid Analysis, in Phospholipids in Nervous Tissues (ed. Eichberg) John Wiley and Sons, pp. 1-44; Hamilton et al. (1992) in the series, A Practical Approach (Rickwood et al. eds.) IRL Press at Oxford University Press; and Kates (1986) Techniques of Lipidology: Isolation, Analysis and Identification in Laboratory Techniques in Biochemistry and Molecular Biology (Burdon et al. eds.) Elsevier.

Phospholipids can also be derived from animal sources. Preferably, the animal is a mammal. Even more preferably, the phospholipids are derived from liver cells. Such phospholipids are commercially available or can be purified from animal tissue by methods known in the art, for instance from animal and egg lecithin or from the compositions described in WO 95/15173.

The phospholipids of the invention can also be synthesized by methods known in the art. Suitable semi-synthetic phospholipids and their synthesis are described in Kates, Techniques of Lipidology (1972).

Various degrees of purity of the phospholipids can be used. Purity can be assayed by any method known in the art such as two dimensional TLC or HPLC and assayed for total lipids, phospholipids or phosphate. Various suitable methods are outlined in Kates (1972). Preferably, the phospholipids must be of sufficient purity such that, when mixed at a total concentration of about 10 mg/mL, the mixture can be sonicated as described below to provide a relatively translucent solution. Preferably, the phospholipids are at least 90% pure, more preferably, they are at least 95% pure and, most preferably, they are at least 99% pure.

In the case of compositions comprising LPA and PEG, where the compositions include other phospholipids, the phospholipids are in the form of Five Phospholipid Mixture. The preferred composition is where phospholipids are combined in a ratio of about 10:10:8:2:4 by weight. A ratio of "about" means that the ratios of the phospholipids can range approximately up to 15% but preferably not more than 5%. More preferably, the ratios are within ± 0.5%.

Other factors which can influence the therapeutic activity of the compositions of the present invention are chain length of the phospholipids, the degree of saturation, presence of cholesterol, presence of micelles, liposomes, detergents, and emulsifying agents, and chain position in LPA, i.e., first or second carbon on the glycerol. While not being bound by any one theory, sonication can cause the formation of liposomes and/or micelles or other multilamellar vesicles, which may enhance activity of the present invention.

3. Other agents

The present invention further encompasses compositions that also comprise a wide variety of other components, as well as methods of using these composition. These

include, but are not limited to, lipids, phospholipids, lipid membrane structures (LMS) and other polymers.

The term "LMS", as used herein, means lamellar lipid particles wherein polar head groups of a polar lipid are arranged to face an aqueous phase of an interface to form membrane structures. Examples of the LMSs include liposomes, micelles, microemulsions, unilamellar vesicles, multilamellar vesicles, and the like.

As used herein, a "liposome" or "lipid vesicle" is a small vesicle bounded by at least one and possibly more than one bilayer lipid membrane. It is made artificially from phospholipids, glycolipids, lipids steroids such as cholesterol, related molecules, or a combination thereof by any technique known in the art, included but not limited to sonication, extrusion, or removal of detergent from lipid-detergent complexes. A liposome can also optionally comprise additional components associated with the outer surface, such as a tissue targeting component. It is understood that a "lipid membrane" or "lipid bilayer" need not consist exclusively of lipids, but can additionally contain any percentage of other components, included, but not limited to, cholesterol and other steroids, proteins of any length, and other molecules, providing the general structure of the membrane is a sheet of two hydrophilic surfaces sandwiching a hydrophobic core. For a general discussion of membrane structure, see *The Encyclopedia of Molecular Biology* by J. Kendrew (1994). For suitable lipids see e.g., Lasic (1993) "Liposomes: from Physics to Applications" Elsevier, Amsterdam.

The lipid bilayer making up the liposome can comprise phospholipids, glycolipids, steroids, and their equivalents; proteins, and lipid-soluble chemicals. Preferably, a composition is chosen that allows the membrane to be formed with reproducible qualities, such as diameter, and is stable in the presence of elements expected to occur where the liposome is to be used, such as physiological buffers and circulating molecules. Preferably, the liposome is resilient to effects of manipulation by storage, freezing, and mixing with pharmaceutical excipients.

Lipids suitable for incorporation into lipid membrane structures include, but are not limited to, natural, semi-synthetic or synthetic mono- or di-glycerophospholipids including phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids, phosphatidylserines, glycero- and cardiolipins. Sphingolipids such as sphingomyelin and cerebrosides can also be incorporated. While natural phospholipids occur with the phospho moiety at the *sn*-3 position and hydrophobic

chains at the *sn*-1 and *sn*-2 positions, synthetic lipids can have alternative stereochemistry with, *e.g.*, the phospho group at the *sn*-1 or *sn*-2 positions. Furthermore, the hydrophobic chains can be attached to the glycerol backbone by acyl, ether, alkyl or other linkages. Derivatives of these lipids are also suitable for incorporation into liposomes. Derivatives suitable for use include, but are not limited to, haloalkyl derivatives, including those in which all or some of the hydrogen atoms of the alkyl chains are substituted with, *e.g.*, fluorine. In addition, cholesterol and other steroids, bolaamphiphiles (lipids with polar moieties at either end of the molecule which form monolayer membranes) and polyglycerolmonoalkylthers can also be incorporated. Liposomes can be composed of a single lipid or mixtures of two or more different lipids.

In one preferred embodiment, the lipid bilayer of the liposome is formed primarily from phospholipids. More preferably, the phospholipid composition is a complex mixture, comprising a combination of phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and sphingomyelin (SM). The LMS can further comprise additional lipids such as phosphatidylinositol (PI), phosphatidylserine (PS), or cardiolipin (diphosphatidylglycerol). If desired, SM can be replaced with a greater proportion of PC, PE, or a combination thereof. PS can optionally be replaced with phosphatidylglycerol (PG). Preferably, at least PC and PE are included; more preferably, at least three of the group PC, PS, PE, and SM are included. The composition is chosen so as to confer upon the LMS both stability during storage and administration.

Practitioners of ordinary skill will readily appreciate that each phospholipid in the foregoing list can vary in its structure depending on the fatty acid moieties that are esterified to the glycerol moiety of the phospholipid. Generally, most commercially available forms of a particular phospholipid can be used. However, phospholipids containing particular fatty acid moieties may be preferred for certain applications.

Preferably, the LMS also includes cholesterol or a related steroid to improve the rigidity of the membrane. Any amount of cholesterol can be used. A preferred ratio of total cholesterol to lipid is between about 0.5 and about 1.2 moles of cholesterol per mole of lipid. More preferred is a molar ratio of about 0.8 to about 1.2:1; even more preferred is a molar ratio of about 0.9 to about 1.1:1; still more preferred is a molar ratio of about 1.0:1.0. Other molecules that can be used to increase the rigidity of the membrane include cross-linked phospholipids.

Other preferred liposomes for use in vivo are those with an enhanced ability to evade the reticuloendothelial system, thereby giving them a longer period in which to reach the target cell. Effective lipid compositions in this regard are those with a large proportion of SM and cholesterol, or SM and PI. Liposomes with prolonged circulation time also include those that comprise the monosialoganglioside GM1 or glucuronide. Liposomes incorporating some proportion of PEG may have a prolonged circulation time. For example, cholesterol can be added at the ratios indicated above to a lipid mixture consisting of any combination of SM, PI, glucuronide, PEG, and other suitable components.

A process for preparing liposomes containing LPA is as follows. An aqueous dispersion of liposomes is prepared from membrane components, such as phospholipids (e.g. phosphatidylcholine, phosphatidylglycerol, sphingomyelin and phosphatidylethanolamine) and glycolipids according to known methods as disclosed. *Ann. Rev. Biophys. Bioeng.* 9:467 (1980). The liposomes can further contain sterols (e.g., cholesterol and cholestanol), dialkylphosphates, diacylphosphatidic acids, stearylamine, α-tocopherol, etc., in the liposomal membrane.

To the liposomal dispersion thus prepared is added an aqueous solution of LPA and the mixture is allowed to stand for a given period of time, preferably under warming at a temperature more than the phase transition temperature of the membrane or above 40°C, followed by cooling to thereby prepare liposomes containing LPA in the liposomal membrane.

Alternatively, the desired liposomes can also be prepared by previously mixing the above-described membrane components and LPA and treating the mixture in accordance with known methods for preparing liposomes.

The lipid vesicles can be prepared by any suitable technique known in the art. Methods include but are not limited to: microencapsulation, microfluidization, LLC method, ethanol injection, freon injection, the "bubble" method, detergent dialysis, hydration, sonication, and reverse-phase evaporation (reviewed in Watwe et al.). For example, ultrasonication and dialysis methods generally produce small unilamellar vesicles; extrusion and reverse-phase evaporation generally produce larger sized vesicles. Techniques can be combined in order to provide vesicles with the most desirable attributes. One particularly preferred method is microfluidization.

The invention encompasses LMSs containing tissue or cellular targeting components. Such targeting components are components of a LMS that enhance its accumulation at certain tissue or cellular sites in preference to other tissue or cellular sites when administered to an intact animal, organ, or cell culture. A targeting component is generally accessible from outside the liposome, and is therefore generally either bound to the outer surface or inserted into the outer lipid bilayer. A targeting component can be *inter alia* a peptide, a region of a larger peptide, an antibody or antigen binding fragment thereof, a nucleic acid, a carbohydrate, a region of a complex carbohydrate, a special lipid, or a small molecule such as a drug, hormone, or hapten, attached to any of the aforementioned molecules.

The LMSs can be targeted to any cell type toward which a therapeutic treatment is to be directed, e.g., a cell type which is undergoing an inappropriate level of apoptosis. Such cells include, but are not limited to, cardiomyocytes, endothelial cells, neuronal cells, hepatocytes, glomerulocytes, lung cells, mucosal cells, skin cells and heart cells.

LMSs can be targeted to such cell types in various ways. For example, a LMS can be modified to contain an antibody, or a fragment of an antibody, specific for a cell surface molecule, or marker, found solely or primarily on a given cell type. Antibodies with specificity toward cell type-specific cell surface markers are known in the art.

The compositions encompassed herein include micelles. The term "micelles" as used herein means aggregates that form from tenside molecules in aqueous solutions above a specific temperature (Krafft point) or a characteristic concentration. This concentration is called the critical micellization concentration, or cmc. When the cmc is exceeded, the monomer concentration remains practically constant and the excess tenside molecules form micelles. They can occur in various shapes (spheres, rods, discs) depending on the chemical constitution of the tenside and on the temperature, concentration or ionic strength of the solution. The micelles have characteristic aggregation numbers with usually only a small distribution spread. Reaching the cmc is manifest by abrupt changes in the surface tension, the osmotic pressure, the electrical conductivity and the viscosity. Micelles are thermodynamically stable association colloids of surfactant substances in which the hydrophobic radicals of the monomers lie in the interior of the aggregates and are held together by hydrophobic interaction; the hydrophilic groups face the water and by solvation provide the solubility of the colloid.

A process for preparing micelles containing LPA is as follows. A micelle-forming surfactant, such as polyoxyethylene sorbitan fatty acid esters, polyoxyethylene castor oil derivatives, polyoxyethylene hardened castor oil derivatives, fatty acid sodium salts, sodium cholates, polyoxyethylene fatty acid ester, and polyoxyethylene alkyl ethers, alkyl glycosides, is added to water at a concentration above the critical micelle concentration to prepare a micellar dispersion. To the micellar dispersion is added an aqueous solution of LPA and the mixture is allowed to stand for a given period of time, preferably under warming at 40°C or higher, followed by cooling, to thereby prepare micelles containing LPA in the micellar membrane. Alternatively, the desired micelles can also be prepared by previously mixing the above-described micelle-forming substances and LPA and treating the mixture according to known methods for micelle formation.

The compositions of the present invention can additionally comprise surfactants. Surfactants can be cationic, anionic, amphiphilic, or nonionic. A preferred class of surfactants are nonionic surfactants; particularly preferred are those that are water soluble. Nonionic, water soluble surfactants include polyoxyethylene derivatives of fatty alcohols, fatty acid ester of fatty alcohols and glyceryl esters, wherein the polyoxyethylene group is coupled via an ether linkage to an alcohol group. Examples include, but are not limited to, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene castor oil derivatives, polyoxyethylene hardened castor oil derivatives, fatty acid sodium salts, sodium cholates, polyexyethylene fatty acid ester and polyoxyethylene alkyl ethers.

Suitable polypeptides include any known in the art and include, but are not limited to, fatty acid binding proteins. Modified polypeptides contain any of a variety of modifications, including, but not limited to glycosylation, phosphorylation, myristylation, sulfation and hydroxylation. As used herein, a suitable polypeptide is one that will protect LPA to preserve its activity. Examples of binding proteins include, but are not limited to, albumins such as bovine serum albumin (BSA) and pea albumin.

Suitable polymers can be any known in the art of pharmaceuticals and include, but are not limited to, naturally-occurring polymers such as dextrans, hydroxyethyl starch, and polysaccharides; and synthetic polymers.

Examples of naturally occurring polymers include proteins, glycopeptides, polysaccharides, dextran and lipids. The additional polymer can be a synthetic polymer. Examples of synthetic polymers which are suitable for use in the present invention include, but are not limited to, polyalkyl glycols (PAG) such as polyoxyethylated polyols (POP)

such as polyoxyethylated glycerol (POG), polytrimethylene glycol (PTG), polypropylene glycol (PPG), polyhydroxyethyl methacrylate, polyvinyl alcohol (PVA), polyacrylic acid, polyethyloxazoline, polyacrylamide, polyvinylpyrrolidone (PVP), polyamino acids, polyurethane and polyphosphazene. The synthetic polymers can also be linear or branched, substituted or unsubstituted, homopolymeric, co-polymers, or block co-polymers of two or more different synthetic monomers.

The synthetic polymers can have the following generic structure:

$$R_1\text{-}(X\text{-}R_2)_a\text{-}R_3$$

where R_1 and R_3 are the same or different and are H, H_3C, OH, R_2 or a reactive group (as described below); where R_2 is a linear or branched substituted or unsubstituted alkyl group; where X is O (in which case the synthetic polymer can be a polyoxyalkylene) or X is NH(C=O) (in which case the synthetic polymer can be a polyamine), or X is absent (in which case the synthetic polymer can be a polyalkylene); and a is an integer between 1 and about 1,000.

Biodegradable polymers can also be included. These include, but are not limited to poly(lactide), poly(glycolide) poly(vinyl alcohol), and crosslinked collagen. The polymers can also include polyglycolic acid, polyethylene terephthalate, polybutyl lactose, polycaprolactone, D-polylactic acid, L-polylactic acid and poly-L-lysine and polymeric mixtures thereof.

Polymers also include polysaccharides. Suitable polysaccharides include, but are not limited to, trehalose, glucose, maltose, lactose, maltulose, iso-maltulose, lactulose, mono-reducing glycosides of polyhydroxy compounds selected from sugar alcohols, other straight chain polyalcohols, raffinose, stachyose, melezitose, dextran, sucrose and sugar alcohols thereof, maltitol, lactitol, iso-maltulose, palatinit, 2-D-glucopyranosyl-1,6-mannitol and their individual sugar alcohols.

The compositions can further include pharmaceutically acceptable excipients. Pharmaceutically acceptable excipients include, but are not limited to, topical pharmaceutically acceptable carrier, cosmetic carrier, sterile solutions, sterile isotonic solutions, ingestable liquids, pharmaceutically acceptable aerosols and solutions for organ/tissue/cell preservation and/or transplantation.

The compositions can further include a pharmaceutically effective agent. Suitable classes of pharmaceutically effective agents include, but are not limited to, drugs, antibiotics, wound healing agents and antioxidants.

Suitable drugs include, but are not limited to, those from the following classes. Other examples are presented in Table 1. Antipyretic and anti-inflammatory drugs, analgesics, antiarthritics, antispasmodics, antidepressants, antipsychotics, tranquilizers, antianxiety drugs, narcotic antagonists, antiparkinsonism agents, cholinergic antagonists, chemotherapeutic agents, immuno-suppressive agents, antiviral agents, parasiticides, appetite suppressants, antiemetics, antihistamines, antimigraine agents, coronary vasodilators, cerebral vasodilators, peripheral vasodilators, hormonal agents, contraceptives, antithrombotic agents, diuretics, antihypertensive agents, cardiovascular drugs, opioids, anti-diarrheal drugs and vitamins are all included in the compositions described herein.

Table 1

Cardiac glycosides	**Immunotherapies**	**Antifungal**
digitalis	interferon	amphotericin B
digitoxin	interleukin-2	myconazole
lanatoside C	monoclonal antibodies	muramyl dipeptide
digoxin	gammaglobulin	clotrimazole
Anticancer	**Steroids**	**Antiarrhythimic**
azathioprine	prednisone	propanolol
bleomycin	triamcinolone	etanolol
byclophosphamide	hydrocortisone	verapamil
adriamycin	dexamethasone	captopril
daunorubicin	betamethosone	isosorbide
vincristine	prednisolone	
Antibiotic	**Hormones**	**Antiviral**
penicillin	antidiuretic	acyclovir and derivatives
tetracycline	corticosteroids	Winthrop-51711
erythromycin	testosterone	ribavirin
cephalothin	estrogen	rimantadine/amantadine
imipenem	thyroid	azidothymidine & derivatives
cefofaxime	growth	adenine arabinoside
carbenicillin	ACTH	amidine-type protease inhibitors
vancomycin	progesterone	
gentamycin	gonadotropin	**Vaccines**
tobramycin	mineralocorticoid	influenza
piperacillin	**Antihistamines**	respiratory syncytial virus
moxalactam	pyribenzamine	Hemophilus influenza vaccine
amoxicillin	chlorpheniramine	
ampicillin	diphenhydramine	**Antihypotension**
cefazolin	**Antiparasitic**	dopamine
cefadroxil	praziquantel	dextroamphetamine
cefoxitin	metronidazole	
other aminoglycosides	pentamidine	
other cephalosporins		

Tabl 1

Antiasthma	Sedatives & Analg sic	Tranquilizers
metaproterenol	morphine	chlorpromazine
aminophylline	dilaudid	benzodiazepine
theophylline	codeine	butyrophenomes
terbutaline	codeine-like synthetics	hydroxyzines
Tegretol	Demerol	meprobamate
ephedrine	oxymorphone	phenothiazines
isoproterenol	Phenobarbital	reserpine
adrenaline	barbiturates	thioxanthines
norepinephrine		
Antihypertensives	**Antidiabetic**	**Other**
apresoline	Diabenese	Receptor agonists and antagonists
etanolol	insulin	

Suitable antibiotics include, but are not limited to, ampicillin, tetracycline, chloramphenicol, erythromycin, amphotericin B and penicillin. Suitable wound healing agents include, but are not limited to, transforming growth factors, platelet-derived growth factors, epidermal growth factors and fibroblast growth factors. Suitable antioxidants include, but are not limited to, naturally-occurring and synthetic antioxidants, such as tocopherols, (e.g., α- tocopherol, vitamin E), ascorbic acid (Vitamin C), beta-carotene (vitamin A), dihydrolipoamide and flavenoids, parahydroxyanisole, butylated hydroxytoluene, butylated hydroxyanisole, Trolox®, propyl gallate, other phenolic antioxidants and phenothiazines; and chelators such as desferrioxamide, HBED and CP130.

The compositions of the present invention can be in either a liquid or solid form. In the liquid form, the LPA and PEG can be concentrated for dilution prior to use. Preferably, the components are in a concentration suitable for immediate use. In the case of a solid, addition of a predetermined amount of an aqueous solution will result in the appropriate concentration of the components. In the case where the composition is a solution incorporating LPA, the LPA is preferably present in an amount of from about 0.00001% to about 10% (weight/volume). More preferably, the LPA is present in an amount of from about 0.001% to about 1% (weight/volume). Most preferably, the LPA is present in an amount of from about 0.005% to about 1% (weight/volume).

Where the composition is a solid incorporating LPA, preferably the LPA is present in an amount of from about 0.00001% to 50% (weight/weight). More preferably, the LPA

is present in an amount of from about 0.001% to 1% (weight/weight). The PEG to LPA weight ratio is preferably 1–100,000:1 and most preferably 10–10,000:1.

The invention further includes methods of making the antiapoptotic compositions.

The phospholipids can be suspended in any buffered solution that is preferably free of divalent cations having a pH range of 2-10, more preferably, about 4-8 and most preferably about 6-8. Suitable buffers include, but are not limited to, D-PBS (phosphate buffered saline, free of calcium and magnesium salts; Gibco BRL) or 50 mM ammonium bicarbonate containing isotonic sodium chloride. When the compositions are to be used therapeutically, the buffered solution is preferably physiologically acceptable. A wide range of pH values are effective. Preferably the pH is between 5.5-8 although, any pH at which the composition is at least minimally effective is suitable for use. The mixture has been found to be most active at pH 8. Preferably, the phospholipids are suspended in 50 mM ammonium bicarbonate in 10 mM sodium chloride, 250 μM EDTA, 10 mM citrate, with a pH of 6.0-8.0.

Preferably, if there is a mixture of phospholipids/lipids, the mixture is dispersed in order to achieve maximal activity. Any method of dispersion that forms particles of about 5–1000 microns is acceptable and about 30-100 microns is preferred. These methods include, but are not limited to, microfluidization, extrusion and sonication, provided that the method does not denature or otherwise chemically modify the phospholipids in such a manner as to render them toxic or of substantially diminished therapeutic activity. Typically, when small batches are prepared the mixture is sonicated until optical clarity is attained although sonication can be continued beyond this point provided that the mixture is not overheated. The preferred sonication parameters are those provided in the examples herein. As used herein, "optical clarity" indicates that the mixture changes from opaque to translucent. This change is readily monitored visually; no further measurements are necessary. However, "translucent" can be defined as when the mixture has an optical density (O.D.) 600 of less than about 0.2 AU.

Concentrations of up to approximately 50 mg/mL phospholipids can be prepared. Preferably, 10 mg/mL solutions are used. Typically, sonication, if used, is in 5 minute alternating cycles, with 5 minutes of sonication followed by 5 minutes of thermal equilibration. However, this can be varied, depending on the volume of mixture being sonicated and the heat generated by sonication.

The total length of sonication depends on the concentration and volume of the mixture being sonicated and the power output of the sonicator. Sonication should proceed until the mixture has become translucent. Typically, mixtures are sonicated for 3 to 90 minutes. Preferably, sonication proceeds by several periods of 5 minutes each, 6 to 12 total periods, with 1 to 5 minutes between each period to allow equilibration and dissipation of heat. The temperature of the water bath should not exceed about 60°C. Preferably, the temperature of the water bath is not allowed to exceed 37°C. Preferably, the sonicated mixture is passed through a sterile filter before use. Preferably, the sterile filter has a 0.22 nm cut off.

The compositions can be sterilized at any point. Sterilization is essential for most of the methods of treatment, although for certain topical applications, it may not be necessary. Preferably, the phospholipid and PEG solutions are made separately, sterilized separately and then combined under aseptic conditions. Alternatively, the phospholipid and PEG can be combined in a single solution, the phospholipid can be dispersed, if necessary, and the combination is sterile filtered.

The compositions can also be prepared and dried to form a solid. The solid is suitable for use once reconstituted. Any method of drying is suitable for use herein, including, but not limited to, freeze-drying, air-drying, spray drying and fluidized bed evaporation.

In one embodiment, the compositions of the present invention are stored under reduced oxygen conditions. Any method of such storage known in the art is suitable for use herein, including, but not limited to, storage under an inert gas such as argon or nitrogen.

The antiapoptotic activity of the compositions of the present invention can be measured in many anti-apoptosis assays known in the art. These include, but are not limited to, the serum deprivation of the C3H/10T½ cell assay described in detail in Example 2 which is the preferred anti-apoptosis assay method. Furthermore, *in vivo* apoptosis inhibition can be measured by any method known in the art. Methods for evaluating therapeutic efficacy in treating an ischemic event are known in the art and described herein.

The therapeutic activity of the compositions described herein, can be measured or determined by any method known in the art. For instance, there are a variety of wound healing assays described in the art and cited herein.

The invention further comprises any of the above-described compositions in combination with compositions described pharmaceutically acceptable vehicle. The level of purity of the components necessary for the composition can be determined empirically and is within the skill of one in the art. The compositions are suitable for use in a variety of disorders, as described below, and in both human and veterinary applications.

III. BIOLOGICAL MATERIALS SUITABLE FOR TREATMENT AND ROUTES OF ADMINISTRATION TO THESE MATERIALS.

Suitable cell types for treatment and/or preservation include, but are not limited to, eukaryotic and prokaryotic cells, such as bacterial cells, plant cells, yeast cells, fungi cells, insect cells, mammalian cells, and human cells in particular. Mammalian cell types encompass cardiomyocytes, endothelial cells, neuronal cells, hepatocytes, renal cells, lung cells, mucosal cells, pancreatic cells, gastrointestinal cells, corneal cells and skin cells. These cell types, and the tissues and organs they form, are suitable for treatment and/or preservation by the methods of the present invention. These cell types can be treated either *in vivo* or *in vitro* using methods of the present invention.

In general, the compositions are pharmaceutically acceptable due to their low toxicity in the therapeutic dosage range, stability and ability to be incorporated into a wide variety of vehicles for numerous routes of administration. The compositions can be administered alone or in combination with other pharmaceutically effective agents including, but not limited to, antibiotics, wound healing agents, antioxidants and other therapeutic agents. Suitable antibiotics include, but are not limited to, ampicillin, tetracycline, chloramphenicol, erythromycin, amphotericin B and penicillin. Suitable wound healing agents include, but are not limited to, transforming growth factors (TGF-αs), epidermal growth factors (EGFs), fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs). Suitable antioxidants include, but are not limited to, naturally-occurring antioxidants such as tocopherols, (e.g., alpha-tocopherol, vitamin E), ascorbic acid (Vitamin C), ß-carotene (vitamin A), dihydrolipoamide and flavenoids; and synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, Trolox®, propyl gallate, other phenolic antioxidants and phenothiazines; and chelators such as desferrioxamide, HBED and CP130.

The compositions contain at least a therapeutically effective amount of at least one of the above-described compositions and at least one physiologically acceptable carrier. A

physiologically acceptable carrier is one that does not cause an adverse physical reaction upon administration and one in which the compositions are sufficiently soluble to deliver a therapeutically effective amount of the compound. The therapeutically effective amount of the compositions depends in part upon the manner of introduction and the indication to be treated and other criteria evident to one of ordinary skill of one in the art. Typically, a therapeutically effective amount is one sufficient to ameliorate or cure the condition being treated as evidenced by diminishment of the symptoms compared to a control. Typically, a therapeutically effective amount is from about 0.0001% or 1 μg/mL by weight of the phospholipid/PEG mixture although a wide range of effective amounts can be used for different indications and can be determined empirically. The route(s) of administration useful in a particular indication are discussed below and are well known to one of skill in the art.

Routes of administration include, but are not limited to, topical, transdermal, parenteral, gastrointestinal, transbronchial, transalveolar, and *in vitro* treatment of cells, tissues or organs followed by in vitro administration of treated cells, tissues or organs. Internal routes of administration encompass any method of *in vivo* administration other than solely by topical application to the skin. Surface administration is accomplished via application of a cream, gel, rinse, etc. containing a therapeutically effective amount of the compositions. Transdermal administration is accomplished by application of a cream, rinse, gel, etc. capable of allowing the active components to penetrate the skin and enter the blood stream. Parenteral routes of administration include, but are not limited to, direct injection such as intravenous, intra-coronary, intra-muscular, intra-peritoneal or subcutaneous injection. Gastrointestinal routes of administration include, but are not limited to, ingestion and rectal. Transbronchial and transalveolar routes of administration include, but are not limited to, inhalation, either via the mouth or intra-nasally (for example, of a mist or a dry powder) and direct injection into an airway, such as through a tracheotomy.

While the compositions can be topically administered alone, it may be desirable to administer them in a mixture with a topical physiologically or cosmetically acceptable carrier. "Topical pharmaceutically acceptable carrier" as used herein is any substantially non-toxic carrier conventionally useable for topical administration of pharmaceutical agents in which the compositions will remain stable and bioavailable when applied directly to skin or mucosal surfaces. For example, the compositions can be dissolved in a liquid,

dispersed or emulsified in a medium in a conventional manner to form a liquid preparation or mixed with a semi-solid (gel) or solid carrier to form a paste, powder, ointment, cream, lotion or the like.

Suitable topical pharmaceutically acceptable carriers include water, petroleum jelly (Vaseline), petrolatum, mineral oil, vegetable oil, animal oil, organic and inorganic waxes, such as microcrystalline, paraffin and ozocerite wax, natural polymers, such as xanthanes, gelatin, cellulose, collagen, starch, or gum arabic, synthetic polymers, such as discussed below, alcohols, polyols, and the like. The carrier can be a water miscible carrier composition that is substantially miscible in water. Such water miscible topical pharmaceutically acceptable carrier composition can include those made with one or more appropriate ingredients set forth above but can also include sustained or delayed release carriers, including water containing, water dispersible or water soluble compositions, such as liposomes, microsponges, microspheres or microcapsules, aqueous base ointments, water-in-oil or oil-in-water emulsions, gels or the like.

In one embodiment of the invention, the topical pharmaceutically acceptable carrier comprises a sustained release or delayed release carrier. The carrier is any material capable of sustained or delayed release of the compositions to provide a more efficient administration resulting in one or more of less frequent and/or decreased dosage of the compositions, ease of handling, and extended or delayed effects on dermatologic conditions. The carrier is capable of releasing the compositions when exposed to any oily, fatty, waxy, or moist environment on the area being treated or by diffusing or by release dependent on the degree of loading of the compositions to the carrier in order to obtain release thereof. Non-limiting examples of such carriers include liposomes, microsponges, microspheres, or microcapsules of natural and synthetic polymers and the like.

Examples of suitable carriers for sustained or delayed release in a moist environment include gelatin, gum arabic, xanthane polymers; by degree of loading include lignin polymers and the like; by oily, fatty or waxy environment include thermoplastic or flexible thermoset resin or elastomer including thermoplastic resins such as polyvinyl halides, polyvinyl esters, polyvinylidene halides and halogenated polyolefins, elastomers such as brasiliensis, polydienes, and halogenated natural and synthetic rubbers, and flexible thermoset resins such as polyurethanes, epoxy resins and the like. Preferably, the sustained or delayed release carrier is a liposome, microsponge, microsphere or gel.

The compositions used in the method of treating dermatologic conditions of the invention are applied directly to the areas to be treated. While not required, it is desirable that the topical composition maintain the active components at the desired location for about 24 to 48 hours, or a length of time sufficient to exert therapeutic efficacy.

If desired, one or more additional ingredients conventionally found in topical pharmaceutical or cosmetic compositions can be included with the carrier, such as a moisturizers, humectants, odor modifiers, buffers, pigments, preservatives, Vitamins such as A, C and E, emulsifiers, dispersing agents, wetting agents, odor-modifying agents, gelling agents, stabilizers, propellants, antimicrobial agents, sunscreens, enzymes and the like. Those of skill in the art of topical pharmaceutical formulations can readily select the appropriate specific additional ingredients and amounts thereof. Suitable non-limiting examples of additional ingredients include superoxide dismutase, stearyl alcohol, isopropyl myristate, sorbitan monooleate, polyoxyethylene stearate, propylene glycol, water, alkali or alkaline earth lauryl sulfate, methylparaben, octyl dimethyl-p-amino benzoic acid (Padimate O), uric acid, reticulin, polymucosaccharides, hydroxyethyl starch (such as, DuPont Pentafraction), hyaluronic acids, aloe vera, lecithin, polyoxyethylene sorbitan monooleate, Vitamin A or C, tocopherol (Vitamin E), alpha-hydroxy of alpha-keto acids such as pyruvic, lactic or glycolic acids, or any of the topical ingredients disclosed in U.S. patents 4,340,586, 4,695,590, 4,959,353 or 5,130,298 and 5,140,043.

Because dermatologic conditions to be treated may be visible, the topical carrier can also be a topical cosmetically acceptable carrier. By "topical cosmetically acceptable carrier" as used herein is meant any substantially non-toxic carrier conventionally useable for topical administration of cosmetics in which the compositions will remain stable and bioavailable when applied directly to the skin surface. Suitable cosmetically acceptable carriers are known to those of skill in the art and include, but are not limited to, cosmetically acceptable liquids, creams, oils, lotions, ointments, gels, or solids, such as conventional cosmetic night creams, foundation creams, suntan lotions, sunscreens, hand lotions, make-up and make-up bases, masks and the like. Thus, to a substantial extent, topical cosmetically acceptable carriers and pharmaceutically acceptable carriers are similar, if not often identical, in nature so that most of the earlier discussion on pharmaceutically acceptable carriers also applies to cosmetically acceptable carriers. The compositions can contain other ingredients conventional in cosmetics including perfumes, estrogen, Vitamins A, C or E, alpha-hydroxy or alpha-keto acids such as pyruvic, lactic or

glycolic acids, lanolin, vaseline, aloe vera, methyl or propyl paraben, pigments and the like.

The effective amount of the compositions used to treat dermatologic conditions or diseases can vary depending on such factors as condition of the skin, age of the skin, the particular ratio of phospholipids or degree of the purity of phospholipids employed, the type of formulation and carrier ingredients used, frequency of administration, overall health of the individual being treated and the like. Those of skill in the dermatologic art taking into consideration these factors and the present disclosure can determine the precise amount for any particular patient use. Preferably the composition is administered in at least two doses and no more than about six doses per day, or less when a sustained or delayed release form is used.

The compositions for topical, oral and parenteral administration usually contain from about 0.001% to about 10% by weight of the PEG Based Molecule compared to the total weight of the composition, preferably from about 0.01% to about 2% by weight of the mixture to the pharmaceutical composition, and especially from about 0.1% to about 1.5% by weight of the mixture to the pharmaceutical composition.

The topical composition is administered by applying a coating or layer to the skin or mucosal area desired to be treated. As a practical matter of convenience, the applied material is rubbed into the area. Applications need not be rubbed into the skin and the layer or coating can be left on the skin overnight.

The present invention provides compositions suitable for transdermal administration including, but not limited to, pharmaceutically acceptable lotions, suspensions, oils, creams, ointments, rinses, gels and liposomal carriers suspended in a suitable vehicle in which a therapeutically effective amount of the compositions has been admixed. Such compositions are applied directly to the skin or incorporated into a protective carrier such as a transdermal device (so-called "patch"). Examples of suitable creams, ointments etc. can be found, for instance, in the Physician's Desk Reference. Examples of suitable transdermal devices are described, for instance, in U.S. Patent No. 4,818,540 (Chien et al.).

The present invention includes compositions suitable for parenteral administration including, but not limited to, pharmaceutically acceptable sterile isotonic solutions. Such solutions include, but are not limited to, saline and phosphate buffered saline for intravenous, intra-muscular, intra-peritoneal, intra-coronary or subcutaneous injection.

The present invention includes compositions suitable for gastrointestinal administration including, but not limited to, pharmaceutically acceptable powders, pills or liquids for ingestion and suppositories for rectal administration.

The present invention includes compositions suitable for transbronchial and transalveolar administration including, but not limited to, various types of pharmaceutically acceptable aerosols for inhalation. An example of a drug administered in the form of an aerosol is pentamidine, which is administered to AIDS patients by inhalation to prevent pneumonia caused by *Pneumocystis carnii*.

In some cases it may be desirable to perform internal delivery of compositions of the present invention in a localized area of the body, organ or tissue. The present invention encompasses methods of delivery including, but not limited to, delivery by catheter inserted into a vessel. Where delivery of the LPA containing compositions is desired to prevent or minimize damage resulting from cardiac ischemia the present invention encompasses intra-coronary delivery by any means, including through a guide catheter.

In some cases it may be desirable to perform internal delivery of LPA containing compositions in a localized area of the body, organ or tissue. The present invention encompasses methods of delivery including, but not limited to, delivery by catheter inserted into a vessel. Where delivery of the PEG Based Molecule containing compositions is desired to prevent or minimize damage resulting from cardiac ischemia the present invention encompasses intra-coronary delivery by guide catheter.

The present invention further encompasses devices suitable for transbronchial and transalveolar administration of the compositions. Such devices include, but are not limited to, atomizers and vaporizers. The present invention also includes devices for electrical or direct injection. Electrical injection, or iontophoresis, is the process of using a small electrical current to drive charged elements, compounds and drugs through the skin to deliver the therapeutic compound to the local tissues or to the whole body without breaking the skin.

The present invention additionally encompasses solutions suitable for flushing, perfusion, and storage of organs and tissues prior to or during transplantation. Such solutions are described in Chien et al. (1993) "Hibernation Induction Trigger for Organ Preservation" in Medical Intelligence Unit, R.G. Landes Co. Austin, TX.

By "organ preservation solution" (OPS) is meant an aqueous solution specifically designed to preserve organs. Preferably the organ is the heart. Preferably, the solutions are used in organ transplantation, but also can be used for flushing the heart during or following ischemia, such as during open-heart surgery. The OPS can also be used to flush the organ to be transplanted either prior to or after harvesting, or both. Preferably, this solution contains between about 0.1% and 20% by weight PEG. More preferably, this solution contains between about 2% and 15% by weight PEG and most preferably, this solution contains between about 7% and about 8% by weight PEG. In one embodiment, this solution contains between about 2% and 15% by weight PEG and can also contain effective amounts of: (a) a buffer such as NaOH, preferably about 30-40 mM, (or sufficient to result in pH of 7.2 to 7.9); (b) an impermeant anion such as Lactobionic acid, preferably about 100 mM; (c) a component serving as a pH buffer, such as KH_2PO_4, preferably about 25 mM; (d) a component providing potassium, such as KOH; and (e) a component controlling cell swelling, such as Raffinose, preferably about 30 mM.

For many applications of the present invention, it is undesirable to have a high level of potassium in the organ preservation solution. Potassium at sufficient doses can cause the beating human heart to become cardioplegic. A "high potassium level" is that concentration that can make a beating human heart cardioplegic. Applications where high potassium levels are not desirable include, for example, the systemic introduction of PEG containing compositions, such as intra-coronary administration to prevent damage to the heart following a period of cardiac ischemia. Preferably, the level of potassium in PEG containing compositions for such applications is below 125 mM, more preferably, the level of potassium is below about 100 mM, and even more preferably, below about 50 mM.

Optionally, the organ preservation solution also contains effective amounts of any component known in the art of organ preservation. These include, but are not limited to, a component controlling redox potential such as glutathione, parahydroxyanisole (PHA), desferoxamine, and nitroglycerin. Glutathione controls the redox potential and protects against free radical injury to cells.

The above-mentioned compositions are meant to describe, but not limit, the compositions suitable for use in the invention. The methods of producing the various compositions are within the ability of one skilled in the art and are not described in detail here.

The methods of producing suitable devices for injection, topical application, atomizers and vaporizers are known in the art and will not be described in detail.

IV. PREVENTION OF APOPTOSIS AND PRESERVATION OR RESTORATION OF CELL, TISSUE AND ORGAN FUNCTION.

The invention further provides methods of treatment comprising administering an amount of the compositions effective to inhibit apoptosis or to protect, preserve or restore cell, tissue or organ function. These methods entail administration of a therapeutically effective amount of the above-described compositions. "Therapeutically effective amount" is an amount sufficient to effect beneficial or desired clinical results. A therapeutically effective amount can be administered in one or more administrations.

Various indications which can be treated, include, but are not limited to, those related to apoptosis, ischemia, traumatic injury and reperfusion damage. Those conditions related to apoptosis include, but are not limited to, dermatological effects of aging, the effects of reperfusion after an ischemic event, immunosuppression, gastrointestinal perturbations, cardiovascular disorders, rejection of tissue transplantation, wound healing and Alzheimer's disease. The treatment can also diminish the apoptosis-related problems associated with immunosuppressing viruses, chemotherapeutic agents, or radiation and immunosuppressive drugs.

The compositions are also suitable for use in organ transplantation during all phases of transplantation. The compositions can be used to prepare the organ by administering an amount of the compositions to the donor effective to stabilize or preserve the organ. The organ can be perfused and/or preserved in OPS solutions containing the compositions. The organ recipient can then be administered an amount of the compositions effective to enhance organ stability and function. The compositions are also particularly suitable for use in treating damage following ischemia and reperfusion whether related to transplantation or other surgical intervention.

Apoptosis related problems are caused by a variety of stimuli, which include, but are not limited to, viruses including, but not limited to, HIV, chemotherapeutic agents, and radiation. These stimuli trigger apoptosis in a variety of disorders, including, but not limited to, those of the digestive tract tissues and associated gastrointestinal perturbations.

Gastrointestinal perturbations include, but are not limited to, damage to the lining of the gut, severe chronic ulcers, colitis, radiation induced damage, chemotherapy induced

damage, and the perturbation of the gastrointestinal tract caused by parasites, and diarrhea from any other cause. Various viral and bacterial infections are known to result in gastrointestinal perturbations; the compositions are also suitable for use in treatment of the side effects associated with these infections. The compositions are particularly suited for use in ameliorating the gastrointestinal disturbances associated with chemotherapy. As shown below, the preferred embodiment of the compositions has over two hundred percent greater antiapoptotic activity compared to the naturally derived, impure phospholipid mixture. Thus, the compositions are suitable for use not only in preventing the diarrhea associated with chemotherapy but also the nausea.

The compositions are particularly suited to treatment of various gastrointestinal conditions in animals, particularly cattle. Such conditions, particularly diarrhea, account for the loss of many calves. Treatment of gastrointestinal conditions is preferably by gastrointestinal administration. In the case of cattle, an effective amount of the compositions can be conveniently mixed in with the feed. In humans, administration can be by any method known in the art of gastrointestinal administration. Preferably, administration is oral.

In addition, the compositions can be administered to immunodeficient patients, particularly HIV-positive patients, to prevent or at least mitigate apoptotic death of T cells associated with the condition, which results in the exacerbation of immunodeficiencies as seen in patients with AIDS. Preferably, administration to such patients is parenterally, but can also be transdermally or gastrointestinally.

The compositions can also be administered to treat apoptosis associated with reperfusion damage involved in a variety of conditions, including, but not limited to, coronary artery obstruction; cerebral or spinal stroke; spinal/head trauma and concomitant severe paralysis; reperfusion damage due to other insults such as frostbite; coronary angioplasty, blood vessel attachment, tissue attachment, such as, without limitation, small bowel attachment, limb attachment, organ attachment and kidney reperfusion.

Myocardial and cerebral infarctions (stroke) are caused generally by a sudden insufficiency of arterial or venous blood supply due to emboli, thrombi, or pressure that produces a macroscopic area of necrosis; the heart, brain, spleen, kidney, intestine, lung and testes are likely to be affected. Cell death occurs in tissue surrounding the infarct upon reperfusion of blood to the area; thus, the compositions are effective if administered at the onset of the infarct, during reperfusion, or shortly thereafter. Thus, the invention includes

methods of treating reperfusion damage by administering a therapeutically effective amount of the compositions to a patient in need of such therapy.

The invention further encompasses a method of reducing the damage associated with myocardial and cerebral infarctions for patients with a high risk of heart attack and stroke by administering a therapeutically effective amount of the compositions to a patient in need of such therapy. Preferably, treatment of such damage is by parenteral administration of the compositions of the invention. Any other suitable method can be used, however, for instance, direct cardiac injection in the case of myocardial infarct. Devices for such injection are known in the art, for instance the Aboject cardiac syringe.

Additionally, the invention provides methods of rapidly normalizing the function of the heart, as measured by electrocardiogram, during or following myocardial ischemia by administering a therapeutically effective amount of a composition of the present invention to a patient in need of such therapy. Again, treatment is preferably by parenteral administration of the compositions of the invention. Suitable alternative methods can be used, such as those methods described above which are used to reduce damage associated with ischemia and reperfusion.

The invention further provides methods of limiting and preventing apoptosis in cells during the culture or maintenance of mammalian organs, tissues, and cells by the addition of an effective amount of the compositions to any media or solutions used in the art of culturing or maintaining mammalian organs, tissues, and cells.

The invention further encompasses media and solutions known in the art of culturing and maintaining mammalian organs, tissues and cells, which comprise an amount of the compositions effective to limit or prevent apoptosis of the cells in culture.

These aspects of the invention encompass mammalian cell culture media comprising an effective amount of at least one composition and the use of such media to limit or prevent apoptosis in mammalian cell culture. An effective amount is one that decreases the rate of apoptosis and/or preserves the cells. The compositions have been found to limit or prevent apoptosis under circumstances in which cells are subjected to mild traumas that would normally stimulate apoptosis. Such traumas can include, but are not limited to, low level irradiation, thawing of frozen cell stocks, rapid changes in the temperature, pH, osmolarity, or ion concentration of culture media, prolonged exposure to non-optimal temperature, pH, osmolarity, or ion concentration of the culture media,

exposure to cytotoxins, disassociation of cells from an intact tissue in the preparation of primary cell cultures, serum deprivation (or growth in serum-free media).

Thus, the invention encompasses compositions comprising tissue culture medium and an effective amount of the compositions. Serum-free tissue culture media to which the compositions can be added as antiapoptotic media supplements include, but are not limited to, AIM V® Media, Neuman and Tytell's Serumless Media, Trowell's T8 Media, Waymouth's MB 752/1 and 705/1 Media, and Williams' Media E. In addition to serum-free media, suitable mammalian cell culture media to which the compositions can be added as antiapoptotic media supplements include, but are not limited to, Basal Media Eagle's, Fischer's Media, McCoy's Media, Media 199, RPMI Media 1630 and 1640, Media based on F-10 & F-12 Nutrient Mixtures, Leibovitz's L-15 Media, Glasgow Minimum Essential Media, and Dulbecco's Modified Eagle Media. Mammalian cell culture media to which the compositions can be added further comprise any media supplement known in the art, including, but not limited to, sugars, Vitamins, hormones, metalloproteins, antibiotics, antimycotics, growth factors, lipoproteins and sera.

The invention further encompasses solutions for maintaining mammalian organs prior to transplantation, which comprise an effective amount of the compositions, and the use of such solutions to limit or prevent apoptosis in such mammalian organs during their surgical removal and handling prior to transplantation. The solutions can be used to flush, perfuse and/or store the organs. In all cases, concentrations of the compositions required to limit or prevent damage to the organs can be determined empirically by one skilled in the art by methods such as those found in the example provided below, as well as other methods known in the art.

It has now been found that the compositions can be topically applied to the skin to treat a variety of dermatologic conditions. These conditions include, but are not limited to, hair loss and wrinkling due to age and/or photo damage. The present invention thus encompasses methods of treating dermatological conditions. Furthermore, hair loss can be caused by apoptosis of the cells of the hair follicles. Therefore, the compositions are suitable for use in topical treatment of the skin to prevent continued hair loss. Stenn et al. (1994) *J. Invest. Dermatol.* 103:107-111.

As discussed above, these conditions are preferably treated by topical application of a composition comprising an effective amount of the compositions. An effective amount of the compositions is one that ameliorates or diminishes the symptoms of the

dermatologic conditions. Preferably, the treatment results in resolution of the dermatologic condition or restoration of normal skin function; however, any amelioration or lessening of symptoms is encompassed by the invention.

V. THE ELECTROCARDIOGRAM

The normal electrocardiogram is composed of a P wave, a QRS complex and a T wave. In the normal electrocardiogram, the Q and S waves are often much less prominent than the R wave and sometimes are absent.

Electrical currents generated as the atria depolarize prior to atrial contraction cause the P wave. Currents generated when the ventricles depolarize prior to their contraction cause the QRS complex. Currents generated as the ventricles recover from the state of depolarization, or repolarize, cause the T wave.

In the normal heart no current flows around the heart when the heart is totally polarized (the T-P interval) or totally depolarized (the S-T interval). Therefore, in the normal electrocardiogram, the T-P and S-T intervals appear level on the electrocardiogram. However, in some cases, injury will prevent the heart from completely repolarizing during the T-P interval. As al result, the potential level of the T-P interval is different from that of the S-T interval. This effect is called the S-T segment shift, and is indicative of damage to the heart muscle.

It has now been found that methods of the present invention prevent or reverse the abnormal heart function represented by the S-T segment shift. The present invention encompasses methods of normalizing the function of the heart during or following myocardial ischemia by treating the heart with a composition comprising PEG or LPA or a combination thereof.

The following examples are provided to illustrate but not limit the invention.

Example 1

Preparation of Five Phospholipid Mixture

Commercially available purified soy phospholipids containing lysophosphatidic acid and the following other phospholipids: PA, PI, LPI, LPC (available, for example, from Avanti® Polar Lipids, Inc.) were suspended in 50 mM ammonium bicarbonate pH 8.0 containing 154 mM NaCl or buffered aqueous solutions free of divalent cations having

a pH range of 5 to 8. Total concentrations of phospholipids of greater than 10 mg/mL can be used provided that clarity is obtainable upon sonication. Total concentrations of up to about 50 mg/mL have been utilized.

Typically, the phospholipid mixtures are suspended in a buffer and the mixture is placed in a disposable borosilicate glass, preferably 1-2 mL in a 16 x 100 mm tube or 0.5-2 mL in a 13 x 100 mm tube, or up to 1 mL in a 12 x 75 mm tube. The combination of phospholipids is then sonicated. Preferably, a small bath sonicator is used, such as that sold by Laboratory Supplies, Hicksville, NY. The temperature of the water bath is between about 21 and 50°C, preferably between about 21°C and about 40°C. The optimal temperature depends on the phospholipids used and can be determined empirically. The water level is adjusted so that it is approximately the same height as the phospholipid mixture in the glass tube(s). Alternatively, a probe sonicator can be used (Fisher Scientific Sonic Dismembrator model 550), as long as care is taken to prevent overheating of the mixture.

The mixture was sonicated for between 3 and 90 minutes, with alternating 5 minute intervals of sonication followed by 5 minutes of thermal equilibration, in a 1-2 mL volume until the mixture became translucent and passed readily through a filter attached to a 5 mL syringe with a pore size of 0.22 μm. Preferably, sonication is for 5-10 minutes. The stability of the compositions at various temperatures was determined. The compositions were stored for one week at 4°C, room temperature, and 65°C. The results show loss of activity after storage at 65°C, while the compositions stored at 4°C or at room temperature do not have a significant loss of activity.

Optimization of each constituent phospholipid was determined by mixing the purified phospholipids in various ratios, varying one phospholipid at a time. Each mixture was analyzed for antiapoptotic activity as described in Example 2. When the apparent optimized ratio was obtained, the ratio of the most active ingredient was varied to find the absolute optimized activity. Table 2 shows the final ratios tested (10:10:8:2:4 is the "Five Phospholipid Mixture" referred to herein).

TABLE 2

PA:	PI:	18:1-LPA:	LPI:	LPC
10:	10:	2 :	2 :	1
10:	10:	2 :	2 :	2
10:	10:	2 :	2 :	4
10:	10:	4 :	2 :	4
10:	10:	4 :	2 :	1
10:	10:	4 :	2 :	2
10:	**10:**	**8 :**	**2 :**	**4**

The concentration of 18:1-LPA was varied as was the chain length to determine the effects of these parameters on activity.

EXAMPLE 2

Phospholipid Mixture in Compositions Containing PEG

Twenty milligrams of Five Phospholipid Mixture stored in $CHCl_3$ (20 mg/mL) under Argon was dried in a glass tube with vacuum concentrator (Savant) with gentle heat. This was sonicated for 5 minutes under an Argon blanket in 1 mL of buffer (50 mM NH_4HCO_3, 104 mM NaCl, 250 μM EDTA) 5 mL of which had been bubbled for 5 minutes with Argon.

250 μL (5 mg) of the Five Phospholipid Mixture sonicate was added under a stream of Argon to 100 mL of 10% PEG20L which had been prepared in water, ultrafiltered then sterilized by filtration (0.22 μM) and stored anaerobically (under Argon in Ar-filled bags). The resulting 50 μg/mL Five Phospholipid Mixture in 10% PEG20L was packaged under a stream of Argon into 10 mL autoclaved amber glass vials in 2 mL and 10 mL amounts and sealed with autoclaved butyl rubber septa/aluminum rings. Prior to use, the septa had been subjected to 2 hours of high vacuum then sealed in an aluminum bag filled with Argon in order to displace as much dissolved oxygen as possible. The Five Phospholipid Mixture/PEG vials were labeled then individually packaged into aluminum bags filled with Argon and stored at 4°C.

EXAMPLE 3

Antiapoptotic Activity Assay

In order to determine the apoptotic activity of compositions of the present invention, the following method of analysis was used. The cell assay is described in detail in U.S. Patent Nos. 5,637,486, 5,620,888, and 5,681,703, and Tomei et al. (1993) *Proc Natl. Acad. Sci.* 90:853-857. Briefly, mouse fibroblast C3H/10T½ cells (clone 8) were

obtained from ATCC (Rockville, MD) and were maintained in exponential growth phase in which the cell cycle is randomly distributed and no cells are arrested in G_0, and in quiescence. Exponential growth phase was assured by seeding at 2000 cells per 1 mL (5 mL for a 60 mm culture plate) five days prior to the beginning of the experiment. Assays were performed on cells only up to passage 15. At T=0, cultures were transferred to serum-free medium, as an apoptosis stimulus, and seed extracts were added. Controls included 10^{-7} and 5 x 10^{-8} M 12-O-tetradecanoyl phorbol-13-acetate (TPA) to ensure the responsiveness of the cell culture. The samples were added to serum free medium and sterile filtered prior to addition to the cultures. Assays were performed in triplicate or quadruplicate. Analyses of the cell responses were made between 18 and 28 hours of serum deprivation. Two assays were performed on each cell culture plate consisting of differential cell counts.

1. All non-adherent or loosely adherent cells were removed from the culture dish and counted by appropriate techniques, typically counting by electronic particle counting instrument. These were the serum deprived released cells (SDR), released by the action of cultivation in serum-free medium. Approximately 95% of these released cells are apoptotic as shown by both ultrastructure analysis and DNA fragmentation analysis.

2. The remaining adherent cells (ADH) were exposed to a buffered, typically pH 7.3, balanced salt solution such as Hanks Balanced Salt Solution without calcium and magnesium salts containing 0.05% trypsin and 0.53 mM ethylene diaminetetraacetic acid (EDTA). Each culture was incubated at either room temperature or 37°C on a rocking platform to ensure uniform distribution of the trypsin reagent over the culture surface. After a standardized period of time, typically 10 minutes, the released cells were removed from each culture dish and measured by the same means as described above, typically electronic particle counting. This ADH cell count is comprised of both trypsin resistant and trypsin sensitive cells as described in U.S. Patent Nos. 5,637,486, 5,620,888, and 5,681,703.

Antiapoptotic activity is expressed in the following examples as the calculated concentration of material (μg/mL of media) required to save 50% of the cells released on serum free treatment.

EXAMPLE 4

Results of in vitro C3H/10T½ Assays

Various compositions were screened in the assay described in Example 3, above. Where phospholipids were screened, the phospholipid mixtures were prepared by combining the desired phospholipids in solution with organic solvent then drying down the mixture with gentle heat (45°C) and high vacuum followed by sonication into aqueous suspension at 5 to 20 mg/mL. The phospholipids were then mixed with serum-free culture medium, which, where desired, contained PEG of the indicated molecular weight, concentration and type, as noted in the Figures and prepared as described above. Figures listing 3 arm and 8 arm PEG refer to the following compositions, respectively: HO-PEG-OCH_2CH(O-PEG-OH)CHO-PEG-OH, and HO-PEG-(CH_2-CH(O-PEG-OH)-CH_2-O)$_5$-CH_2-CH(-O-PEG-OH)- CH_2-O-PEG-OH, both of which are commercially available from Shearwater Polymers, Inc. The results obtained are depicted in Figures 1-3. It is clear from these results that both linear and branched PEG Based Molecules have anti-apoptotic activity.

EXAMPLE 5

In vitro Cardiomyocyte Assays

Isolation of rat neonatal cardiomyocytes. Cardiomyocytes were prepared from hearts of day-old Sprague Dawley rats by trypsinization and mechanical disaggregation (Simpson, (1985)) Circ. Res. 56:884–894. The cells were resuspended in MEM, 1xMEM vitamins (Gibco), 5% fetal bovine serum and 50 U/mL penicillin-G and pre-plated for 30 minutes to reduce contamination of non-myocytes. The non-adherent cardiac myocytes were separated and seeded in 2 mL in 35 mm dishes at a density of $3.5x10^5$ viable cells per mL. The cells were allowed to adhere for 16-24 hours in a 37°C/5% CO_2 humidified incubator.

Cardiomyocyte treatment

For serum deprivation, the medium was replaced with fresh serum-free RPMI, whereas serum/glucose deprivation was performed using glucose-free RPMI. The induction of cell death by Adriamycin or C_2-ceramide was accomplished by the addition of the agent prepared in serum-free RPMI. As a model of ischemia, cultures in serum and glucose free RPMI were placed in an airtight chamber and the latter was continuously perfused with oxygen-free gas overlay of 95% N_2/5% CO_2 for 8 hours at 37°C. To model

reperfusion of the ischemic cells, 10% fetal bovine serum, 2 g/L of glucose were added and the cultures were returned to a normal oxygen gas overlay (37°C/5% CO_2) in a humidified incubator for 16 hours.

To investigate effects of PEG, LPA or PEG plus LPA on cell death, various combinations of these components were added to cells in serum free medium at the beginning of cytotoxic treatment.

Measurement of cell death

Because cardiomyocytes are terminally differentiated non-dividing cells, viability was determined by measurement of the decrease in the relative number of adherent cells. The measurement of non-adherent cells was found to be less reproducible because of their rapid lysis following release from adhesion substrate. Adherent cardiomyocytes were collected from culture dishes using 0.25% Trypsin/0.05% EDTA and counted on Coulter Counter ZM and Coulter Channelyzer 256.

1. The results were as follows:

2. 0.3-2% PEG with molecular weight 20,000 kilo Dalton (kDa) enhances the protective effect of Five Phospholipid Mixture against serum/glucose deprivation induced cell death (Fig.4).

3. At concentrations of 1% and higher, 20,000 Dalton PEG prevents cardiomyocyte death induced by serum/glucose deprivation (Fig. 5).

4. Linear PEGs with molecular weight 200-6,000 Dalton did not protect cardiomyocytes from serum/glucose deprivation induced cell death in this assay. Linear PEG 8,000 showed protection of cardiomyocytes. Significant protection of cardiomyocytes is seen with molecular weights of PEGs above 8,000. (Fig. 6).

5. Mixtures of PEG with Five Phospholipid Mixture and 18:1-LPA equally protect cardiomyocyte death induced by ischemia and reperfusion (Fig. 10).

It is clear from these results that PEG has anti-apototic activity as shown in the cardiomyocyte assay. Additionally, the presence of PEG improves the antiapoptotic activity of LPA and LPA containing compositions.

EXAMPLE 6

Evaluation of Infarct Measurement and EKG Alterations in a Regional Ischemic Pig Heart Model

This example evaluates the antiapoptotic activity of PEG20L in a regional ischemic pig heart model. Yucatan Miniature pigs (approx. 30 kg each) were treated with general anesthesia followed by balloon catheterization to create an occlusion of the first obtuse marginal branch of the circumflex coronary artery. After 105 minutes of occlusion treatment with control, infusion of PEG20L (100 mg/mL), or PEG20L and Five Phospholipid Mixture (100 mg/mL PEG + 50 μg/mL Five Phospholipid Mixture) solution was started through the guide catheter into the aortic root in close approximation to the occlusion. The solutions were introduced initially by a bolus equal to 25% of the hourly drug dose, followed by continuous infusion through the guide catheter for 135 minutes. The occlusion was removed after 120 minutes (15 minutes after the start of drug delivery).

Heart function was monitored by EKG and cardiac output was determined by contrast imaging. The animal was euthanized 1-3 days after the occlusion. The heart was removed for histology and examined. Infarct size was determined by a method of triphenyl tetrazolium chloride (TTC) staining for the area at risk and actual infarct. The area not at risk was stained with a blue pigment or Evan's Blue dye.

Pigs were treated with one of the following test regimens (displayed as total dose):

a) 250 mg/kg PEG20L only (10% ultrafiltered in water) (n = 2);

b) 125 μg/kg Five Phospholipid Mixture + 250 mg/kg PEG20L (n = 3).

Four additional pigs were occluded but not treated with any solution, for use as controls.

Results appear in Figure 8. Of the four controls the mean infarct size (as % risk zone) was 34.5% ± 7.2 %. The controls also showed a marked unresolved S-T segment shift during the ischemic period that reflects severe myocardial ischemia and infarction.

In contrast to the controls, two effects were noted in the treatment groups. First, in some animals, within 2 minutes of the start of drug infusion there was a normalization of the EKG signal disturbances in the occluded hearts. During the ischemic period the normal EKG signal shifted to one in which there was an elevation in the S-T segment indicating an impairment of myocyte repolarization. This elevation disappeared in several pigs treated with PEG20L and Five Phospholipid Mixture. Some pigs that were treated were not included in the infarct measurement data due to technical difficulties in

determining the infarct size. However, some of these animals did exhibit a reduction in S-T segment elevation.

The second effect noted was a decrease in infarct size in the treated animals when compared to the control animals (Fig. 8).

It is clear from this example that PEG and PEG in combination with LPA are effective therapeutics for the treatment of cardiac ischemia, as both protect tissue from infarct damage and reduce the level of S-T segment shift measured by electrocardiogram.

EXAMPLE 7

Regional ischemic dog heart model

The experimental model used was a regional ischemic dog heart (mongrel male hound, approximately 20 kg) with the left anterior descending coronary artery clamped distal to the first diagonal branch rendering a portion of the left ventricle ischemic. The clamp was maintained for 90 minutes then released gradually over a 5-minute period. A narrow canula was placed into the left main coronary artery through which Five Phospholipid Mixture (5 mL of 50 μg/mL in 10% PEG) was infused starting 75 minutes after the start of the ischemic period, and continuing for 75 minutes in total (approximately 67 μL/min., approximately 3.3 μg Five Phospholipid Mixture/min.). The total dose was 250 μg Five Phospholipid Mixture and 500 mg PEG20L.

Determination of Infarct size

After staining and fixation, the atria and right ventricle were removed from the left ventricle. After removal of the apex, the portion of the left ventricle distal to the mitral apparatus was cut into 5 transverse sections of equal thickness. Evans Blue dye injected into the circumflex vessel stained the circumflex territory blue. TTC stained viable LAD territory red, while the infarcted myocardium remained unstained (white). Sections were weighed and photographed for documentation and future verification of results. Computer assisted planimetry was used to measure the areas of the circumflex, viable LAD and infarcted LAD territories. Calculation of infarct size was based upon the assumption that the area of infarction in the sectioned plane was representative of the mass of infarcted tissue in that plane.

Study Protocols

1. Study-drug group; dogs were treated with Five Phospholipid Mixture 50 μg/mL and PEG 100 mg/mL as an infusion (at 4 mL/hr) starting 15 min before reperfusion and going on for 60 min into the reperfusion period.

2. Placebo group: dogs received placebo following the same modalities described for the study drug group

3. SOD-Catalase group: dogs received superoxide dismutase (SOD) plus catalase following the same modalities described by Simpson et al. (1987) Fed. Proc. 46:7, 2413–21.

The rationale for using a third group of dogs in the present protocol resided in the fact that the SOD + Catalase group represents a positive control. It has been shown that the combination of oxygen radical scavenger is very efficacious in limiting myocardial reperfusion injury in the canine model.

The results obtained in the dog heart experiments are depicted in Figure 9. It is clear that the combination of PEG and Five Phospholipid Mixture provide significant protection against ischemia and reperfusion damage.

EXAMPLE 8

Evaluation of Infarct Measurement in a Regional Ischemic Rabbit Heart Model

Male New Zealand white rabbits were initially anesthetized using a mixture of ketamine (400 mg per rabbit) and xylazine (20 mg per rabbit) administered intramuscularly in two doses, approximately 10 minutes apart. Throughout the study, a level of deep anesthesia is maintained using sodium pentobarbital given intraperitoneally at a dose of approximately 50 mg/hour. All rabbits were intubated and mechanically ventilated using room air supplemented with oxygen. Fluid filled catheters were placed into the jugular vein to administer fluids. A catheter was also placed into the left carotid artery to measure heart rate and blood pressure and to obtain reference blood samples during regional myocardial blood flow measurements. The chest was opened through the left fourth intercostal space. Then, the pericardium was incised and the heart was exposed. A large anterolateral branch of the circumflex artery, or the circumflex artery itself was identified and encircled with a 4-0 silk suture. The ends of the suture were threaded through a piece of flanged tubing, forming a snare, which was later used to occlude the artery. A catheter was then placed into the left atrial appendage to inject the control or test solution, the radioactive microspheres and blue pigment at the conclusion of the procedure.

After the surgical preparation, the rabbits were stabilized for ten minutes. At this time, the rabbits were randomized into either the treatment or control group. The treatment solution contained 1 mg/mL of the Five Phospholipid Mixture, described in Example 3, above, 8 mg/mL PEG 20L, in 10 mM citrate 110 mM NaCl. The control solution contained 10 mM citrate and 110 mM NaCl. Five minutes before occlusion, a bolus dose of 1 mg/kg body weight of either the Five Phospholipid Mixture/PEG solution or the control solution was administered into the left atrium. Immediately following the bolus dose, infusion was started at the rate of 1 mg/kg for one hour into the left atrium. Baseline hemodynamics and core body temperature were recorded. Next, the artery was occluded for 30 minutes. Following this, the hearts were reperfused for three hours. Heart rate and arterial blood pressure were recorded at 20 minutes of occlusion and at time points during reperfusion at a 25 mm/second paper speed. Regional myocardial blood flow measurements were performed on two occasions; during coronary occlusion (20 minutes) to confirm no blood flow in the ischemic zone, and during early reperfusion (30 minutes) to confirm reflow in the same zone. At the end of three hours of reperfusion, the coronary artery is reoccluded. Next, 4 mL of 50% Unisperse blue (Ciby-Geigy, Hawthorne, NY) were infused through the left atrial catheter and allowed to circulate throughout the vascular system. The rabbit was then euthanized by an overdose intravenous injection of xylazine (300 mg) followed by 12 mEq of potassium chloride given into the left atrium. Prospective exclusion criteria included an ischemic risk zone of less than 10% of the left ventricular weight, a regional blood flow of more than 0.2 mL/minute/g in the risk zone during coronary artery occlusion (lack of ischemia), or a regional blood flow of less than 0.4 mL/min/g in the risk zone at 30 minutes of reperfusion (failure to reperfuse).

Infarct size was evaluated as follows. The right ventricle was trimmed off and the left ventricle was sliced transversely into seven or eight sections, approximately two millimeters in thickness. These slices were photographed to identify the ischemic risk regions (uncolored by the blue pigment) and the non-ischemic regions (colored by the blue pigment). The slices were then incubated in a 1% solution of triphenyltetrazolium chloride pre-heated to 37 °C for 10 minutes and rephotographed for analysis of area of necrosis. All sections were later fixed in formalin. These photographic slides were projected and areas of risk (AR) and areas of necrosis (AN) were traced by planimetry. The planimetered areas of each slice were multiplied by the weight of the slice and then

summed. Because infarct size is measured from photographic slides, the entire left ventricle was used for the analysis.

Regional myocardial blood flow (RMBF) was measured as follows. Just before measuring RMBF, during occlusion, the atrial catheter was disconnected from the treatment infusion pump. Radioactive microspheres were injected via the atrial catheter. The catheter was then reprimed with approximately 0.2 mL of the drug treatment, and the catheter was reconnected to the pump and infusion continued.

Regional myocardial blood flow was measured with 11 μm radioactive microspheres labeled with ^{141}Ce, ^{96}Nb or ^{103}Ru (New England Nuclear, North Billerica, MA), using approximately 500,000 per injection. These microspheres were injected into the left atrial catheter. At the same time, a reference blood sample was obtained from the carotid artery at 2.06 mL/minute. The blood removed during RMBF measurement was about 5 mL. These volume changes do not cause changes in systemic arterial pressure. At the end of the protocol, after the photographic slides had been taken and the heart weight, myocardial samples were cut from the center of the non-ischemic and the ischemic regions, weighed and counted with the reference blood samples in a well gamma counter. Blood flows at each interval, for ischemic and non-ischemic tissues, were then computed and expressed in mL/minute/g.

The results were as follows. With a risk zone of approximately 25% of the left ventricle, treatment with the control solution resulted in infarct size of approximately 40% of the risk zone on average, as compared to an average of only approximately 25% of the risk zone in the subjects treated with the PEG plus Five Phospholipid Mixture.

EXAMPLE 9

Preparation of PEG-Cardiosol

An OPS of the present invention is substantially produced as described in United States Patent No. 4,938,961, issued July 3, 1990, to Collins et al., with the following or additional modifications: A solution comprising the following ingredients is prepared:

(a) NaOH, about 5-125 mM;

(b) Lactobionic acid, about 80-120 mM;

(c) KH_2PO_4, about 5-50 mM;

(d) KOH, about 5-125 mM;

(e) Raffinose, about 25-35 mM;

(f) $MgSO_4$, about 4-6 mM; and

(g) optionally, PHA, about .04 mM;

(h) optionally, Glutathione, about 3 mM.

A composition comprising PEG, having an average molecular weight of greater than approximately 8,000 Daltons, preferably in the range of greater than approximately 8,000 to approximately 100,000 Daltons, and most preferably approximately 20,000 Daltons, is stored anoxically in the absence of UV light at a low temperature as described herein.

The composition comprising PEG can be dissolved in water and ultrafiltered or dialysed as described herein. Evaluation of ionic content, antioxidant content and aldehyde levels, or any combination of these tests, can be performed to determine the level of decomposition products and other undesirable components in the PEG. In one embodiment of the present invention, PEG solutions that contain more than 1.0 mole-percent aldehyde according to the aldehyde assay described herein are treated by ultrafiltration or dialysis to reduce levels of contaminants.

Upon a demonstration from these methods that the PEG is suitable for the present invention, PEG (preferably at a final concentration of between about 0.1% and about 20%, more preferably between about 2% and about 15%, even more preferably, about 7 - 8 %, most preferably about 7.5 % by weight) is added to the solution. Optionally, PHA, desferoxamine, or nitroglycerine, or any combination of these, can be added. The solution is then stored under anoxic conditions as described above.

Direct testing of the ability of various OPS compositions to preserve organs can also be performed on animal organs, as is well known in the art and described herein. Cardiac Output (CO) and organ failure rates are typically measured. See, for example, Wicomb et al. *Transplantation* 47:733-734 (1989) and documents cited herein.

The PEG-Cardiosol solution described in this example can be used for organ preservation as a perfusate in a process in which the perfusate is flowed through the circulatory system of the organ, and can also be used for cardioplegia applications.

EXAMPLE 10

The Effects of Substituting PEG20L for PEG20M in a heart preservation solution

The effects of the substitution of PEG20L for PEG20M in a heart preservation solution was tested. Rabbit hearts were treated and Cardiac Output (CO) measured as

described previously in Wicomb et al. *J. Surg. Res.* 40:276 (1986); and Wicomb et al. *Transplantation* 48:6-9 (1989), and as described in Table 3, below.

TABLE 3

CARDIAC OUTPUT (mL/min)

Solution used	Flush Only (0 Hour)	4 Hour	24 Hour
PEG20M-Cardiosol	171±17 n=14	164±31 n=8	212±48 n=10
PEG20L-Cardiosol	312±24 n=4	327±30 n=20	330±32 n=4
UW solution	282±5 n=4	256±65 n=12	88±32 n=2
No solution	285±32 n=4		

60 minute cardiac output (mL/min) data following zero, 4 and 24 hours ice storage of rabbit hearts using PEG20M-Cardiosol, PEG20L-Cardiosol, University of Wisconsin (UW) solution or no solution. n indicates the number of rabbit hearts used.

The data clearly indicate that the use of PEG20L-Cardiosol substantially improves the cardiac output of rabbit hearts compared to either of the other two solutions, following 24 hours ice storage.

Additionally, these solutions were tested at varying concentrations, as shown in Table 4, below.

TABLE 4

CARDIAC OUTPUT (mL/min)

Solution used	5%	8%	10%
PEG20M-Cardiosol	136±19.4 n=13	NT	212±8 n=30
PEG20L-Cardiosol	NT	256±12.3 n=44	NT

NT indicates that the solution was not tested. "n" indicates the number of rabbit hearts tested. Cardiac output was tested after 60 minutes on the working heart column following 24-hour storage on ice, as described in Wicomb et al. *J. Heart Lung Transplantation* 13:891-894 (1994).

The data clearly indicate that the use of 8% PEG20L in an OPS improves the cardiac output of rabbit hearts compared to PEG20M-Cardiosol comprising 5% or 10% PEG20M.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the descriptions and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

Claims

We claim:

1. A method of protecting, preserving or restoring function in cells, tissue or organs, comprising administering a therapeutically effective amount of a pharmaceutically acceptable composition comprising a PEG Based Molecule, wherein said pharmaceutically acceptable composition does not contain enough potassium to cause cardioplegia in a patient's beating heart.

2. The method of claim 1, wherein said PEG Based Molecule has the formula R_1O-$(CH_2CH_2O)_n$-R_3, wherein 'n' is an integer between 1 and about 1,000 wherein said PEG Based Molecule comprises at least 4 ethylene oxide monomers, and wherein R_1 and R_3 are independently selected from the group consisting of H, CH_3, unsubstituted linear alkyl, substituted linear alkyl, unsubstituted branched alkyl and substituted branched alkyl.

3. The method according to claim 2, wherein R1 is H and R3 is selected from the group consisting of linear alkyl and branched alkyl.

4. The method according to claim 2, wherein R1 is H and R3 is H.

5. The method according to claim 3, wherein R_3 is linear alkyl.

6. The method according to claim 3, wherein R_3 is branched alkyl.

7. The method according to claim 4, wherein 'n' is an integer between about 200 and about 1000.

8. The method according to claim 5, wherein the linear alkyl group is selected from the group consisting of $-CH_2CH_2CH_2CH_2Ar$, $-CH_2CH_2CH(Ar)R_4$ and $-CH_2CH(O(CH_2CH(CH_3)O)_nR_4)CH_3$, wherein R_4 is selected from the group consisting of H, CH_3, linear alkyl, branched alkyl, aryl and substituted aryl.

9. The method according to claim 5, wherein the linear alkyl group is selected from the group consisting of $-CH_2C(O)OR_4$, $-CH_2C(O)NR_5R_6$, $-CH_2CH_2C(O)OR_4$, $-CH_2C(O)NR_5R_6$, and wherein R_4, R_5 and R_6 are independently selected from the group consisting of H, CH_3, linear alkyl, branched alkyl, aryl and substituted aryl.

10. The method according to claim 6, wherein the branched alkyl group is selected from the group consisting of $-CH(CH_2OR_4)CH_2OR_7$, $-CH_2CH(CH_2CH_2OR_4)_2$ and $-CH_2CH_2CH(CH_2OR_4)CH_2CH_2OR_4$, wherein R_4 and R_7 are independently selected from the group consisting of H, CH_3, linear alkyl, branched alkyl, aryl and substituted aryl.

11. The method according to claim 8, wherein the PEG Based Molecule is a Star PEG.

12. The method according to claim 8, wherein the PEG Based Molecule is a co-polymer.

13. The method according to claim 12, wherein the PEG Based Molecule is a polyethylene glycol-polypropylene glycol copolymer.

14. The method according to claim 10, wherein the PEG Based Molecule is a Branched PEG Based Molecule.

15. The method according to claim 11, wherein the Star PEG has up to approximately 100 arms.

16. The method according to claim 14, wherein the Branched PEG Based Molecule has 3, 4, 6 or 8 arms.

17. The method according to claim 1, wherein said composition is administered to a patient suffering from a gastrointestinal perturbation.

18. The method according to claim 17, wherein the gastrointestinal perturbation is caused by a stimulus selected from the group consisting of human immunodeficiency virus, chemotherapeutic agents and radiation and those associated with infectious diseases.

19. The method according to claim 18, wherein the gastrointestinal perturbation is due to inflammatory bowel disease.

20. The method according to claim 18, wherein the infectious disease is selected from the group consisting of diarrhea-causing organisms.

21. The method according to claim 18, wherein said composition decreases immunodeficiencies associated with immunosuppressing viruses, chemotherapeutic agents, or radiation and immunosuppressive drugs.

22. The method according to claim 21, wherein the virus is human immunodeficiency virus.

23. The method according to claim 1, wherein said composition is administered to a patient who is undergoing or has recently undergone ischemia and/or reperfusion subsequent to ischemia.

24. The method according to claim 23, wherein the reperfusion is associated with coronary artery obstruction; cerebral stroke; spinal stroke; spinal/head trauma; frostbite; coronary angioplasty; blood vessel attachment; tissue attachment; limb attachment; tissue attachment; bypass surgery; organ attachment; or kidney reperfusion.

25. The method according to claim 1, wherein the composition is administered to a patient suffering from a dermatologic condition.

26. The method according to claim 25, wherein administration comprises topically administering a therapeutically effective amount of said pharmaceutically acceptable composition.

27. The method according to claim 26, wherein the dermatological condition is wrinkling, sagging, psoriasis, baldness or hair loss.

28. The method according to claim 1, wherein said composition further comprises an LPA.

29. The method according to claim 1, wherein said composition contains less than 125 μM potassium.

30. The method according to claim 1, wherein the composition is administered to a patient suffering from a wound.

31. A method of treating apoptosis, preserving or restoring function in cells, tissue or organs, comprising administering a therapeutically effective amount of a pharmaceutically acceptable composition comprising a Branched PEG Based Molecule, wherein said Branched PEG Based Molecule does not decompose to release a phenol based compound.

32. The method according to claim 31, wherein said Branched PEG Based Molecule comprises between about 200 and about 1000 ethylene oxide monomers.

33. The method according to claim 32, wherein the Branched PEG Based Molecule is a Star PEG.

34. The method according to claim 33, wherein the Star PEG has up to approximately 100 arms.

35. The method according to claim 31, wherein the Branched PEG Based Molecule has between about 3 and about 8 arms, inclusive.

36. The method according to claim 31, wherein said composition is administered to a patient suffering from a gastrointestinal perturbation.

37. The method according to claim 36, wherein the gastrointestinal perturbation is caused by a stimulus selected from the group consisting of human immunodeficiency virus, chemotherapeutic agents and radiation and those associated with infectious diseases.

38. The method according to claim 37, wherein the gastrointestinal perturbation is due to inflammatory bowel disease.

39. The method according to claim 37, wherein the infectious disease is selected from the group consisting of diarrhea-causing organisms.

40. The method according to claim 37, wherein said composition decreases immunodeficiencies associated with immunosuppressing viruses, chemotherapeutic agents, or radiation and immunosuppressive drugs.

41. The method according to claim 40, wherein the virus is human immunodeficiency virus.

42. The method according to claim 31, wherein said composition is administered to a patient who is undergoing or has recently undergone ischemia and/or reperfusion subsequent to ischemia.

43. The method according to claim 42, wherein the reperfusion is associated with coronary artery obstruction; cerebral stroke; spinal stroke; spinal/head trauma; frostbite; coronary angioplasty; blood vessel attachment; tissue attachment; tissue attachment; bypass surgery; limb attachment; organ attachment; or kidney reperfusion.

44. The method according to claim 31, wherein the composition is administered to a patient suffering from a dermatologic condition.

45. The method according to claim 44, wherein administering comprises topically administering a therapeutically effective amount of said pharmaceutically acceptable composition.

46. The method according to claim 45, wherein the dermatological condition is wrinkling, sagging, psoriasis, baldness or hair loss.

47. The method according to claim 31, wherein said composition further comprises a phospholipid.

48. The method according to claim 47, wherein said phospholipid is a lysophosphatidic acid.

49. The method according to claim 31, wherein the composition is administered to a patient suffering from a wound.

50. The method according to claim 31, wherein said composition further comprises a tissue culture media.

51. The method according to claim 50, wherein said composition is administered to cells selected from the group consisting of bacterial cells, plant cells, yeast cells, fungi cells, insect cells, and mammalian cells.

52. The method according to claim 50, wherein said composition is administered to human cells.

53. The method according to claim 52, wherein the cells are part of a tissue or organ.

54. A method of preserving or restoring function in cells, tissue or organs comprising administering a therapeutically effective amount of a pharmaceutically acceptable aqueous solution, consisting essentially of a PEG Based Molecule.

55. A method of preserving or restoring function in cells, tissue or organs, comprising administering a therapeutically effective amount of a pharmaceutically acceptable composition comprising a Linear PEG Based Molecule to a patient in need of such treatment, wherein said Linear PEG Based Molecule has a molecular weight greater than 8,000 Daltons.

56. The method according to claim 55, wherein the patient is suffering from a gastrointestinal perturbation.

57. The method according to claim 56, wherein the gastrointestinal perturbation is caused by a stimulus selected from the group consisting of human immunodeficiency virus, chemotherapeutic agents and radiation and those associated with infectious diseases.

58. The method according to claim 56, wherein the gastrointestinal perturbation is due to inflammatory bowel disease.

59. The method according to claim 56, wherein the infectious disease is selected from the group consisting of diarrhea-causing organisms.

60. The method according to claim 57, wherein said treatment decreases immunodeficiencies associated with immunosuppressing viruses, chemotherapeutic agents, or radiation and immunosuppressive drugs.

61. The method according to claim 60, wherein the virus is human immunodeficiency virus.

62. The method according to claim 55, wherein the patient is undergoing or has recently undergone ischemia and/or reperfusion subsequent to ischemia.

63. The method according to claim 62, wherein the reperfusion is associated with coronary artery obstruction; cerebral stroke; spinal stroke; spinal/head trauma and concomitant severe paralysis; frostbite; coronary angioplasty; blood vessel attachment; tissue attachment; limb attachment; organ attachment; bypass surgery; or kidney reperfusion.

64. The method according to claim 55, wherein the patient is suffering from a dermatologic condition.

65. The method according to claim 64, wherein administering comprises topically administering a therapeutically effective amount of said pharmaceutically acceptable composition.

66. The method according to claim 65, wherein the dermatologic condition is wrinkling, sagging, psoriasis, baldness or hair loss.

67. The method according to claim 55, wherein said composition further comprises a phospholipid.

68. The method according to claim 55, wherein said phospholipid is a lysophosphatidic acid.

69. The method according to claim 55, wherein the patient is suffering from a wound.

70. The method according to claim 55, wherein said Linear PEG Based Molecule comprises PEG20L.

71. The method of treating apoptosis, preserving or restoring function in cells *in vitro*, comprising treating said cells with a composition comprising a tissue culture media and an effective amount of a PEG Based Molecule.

72. The method according to claim 71, wherein the origin of the cells is selected from the group consisting of: bacterial cells, plant cells, yeast cells, fungi cells, insect cells, and mammalian cells.

73. The method according to claim 72, wherein the origin of the cells is human.

74. The method according to claim 72, wherein the cells are part of a tissue or organ.

75. A method of organ preservation comprising adding an effective amount of a composition comprising a PEG Based Molecule to the solution in which the organ is stored, wherein said solution does not contain enough potassium to cause cardioplegia in a beating human heart.

76. A method of organ preservation comprising administering to the host animal at least one intravenous bolus of an effective amount of a composition comprising a PEG Based Molecule, wherein said composition does not contain enough potassium to cause cardioplegia in a beating human heart.

77. A method of treating cardiac ischemia comprising administering a therapeutically effective amount of a pharmaceutically acceptable composition comprising a PEG Based Molecule to a human or animal in need of such treatment, wherein said pharmaceutically acceptable composition does not contain enough potassium to cause cardioplegia in a beating human heart.

78. The method of claim 77, wherein said treatment protects tissue from infarct damage.

79. A method of treating cardiac pathology associated with ST segment shift as measured by electrocardiogram, comprising: administering a therapeutically effective amount of a pharmaceutically acceptable composition comprising a PEG Based Molecule to a patient suffering from or at risk of suffering from ST segment shift.

80. The method of claim 79, wherein said PEG Based Molecule comprises PEG20L.

81. A method of protecting, preserving or restoring function in cells, tissue or organs, comprising administering a therapeutically effective amount of a pharmaceutically acceptable composition comprising a PEG Based Molecule and a lysophosphatidic acid (LPA).

82. The method of claim 81, wherein said PEG Based Molecule comprises a Linear PEG Based Molecule.

83. The method of claim 81, wherein said PEG Based Molecule comprises a Branched PEG Based Molecule.

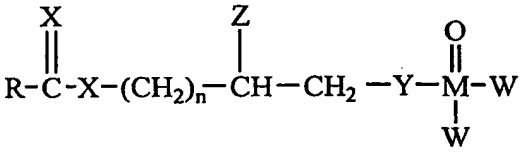
84. The method of claim 83, wherein said PEG Based Molecule comprises PEG20L

85. A pharmaceutical composition for the treatment of myocardial infarction comprising therapeutically effective quantities of a PEG Based Molecule and a lysophosphatidic acid (LPA).

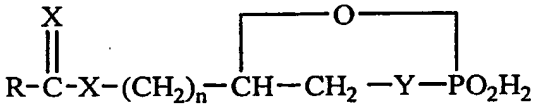
86. A method of treating myocardial infarction which comprises administering a therapeutically effective quantity of the composition of claim 85.

87. A composition comprising lysophosphatidic acid (LPA) and a PEG Based Molecule.

88. The composition according to claim 87, wherein the LPA has the formula:



or a cyclic phosphate derivative thereof having the structure:



wherein

each X is independently O or S;

M is P or S, where when M is S, one W is (=O);

each W is independently SH, OH, $OCH_2CH(NH_2)CO_2H$, $OCHCH_3CH(NH_2)CO_2H$, OPO_3H_2, or $OPO_2HOPO_3H_2$, where if one W is OPO_3H_2 or $OPO_2HOPO_3H_2$, the remaining W is OH;

Z is OH, SH, NH_2, halogen, OPO_3H_2, H, $O(CH_2)_bCH_3$ where b=0 to about 2, or SO_3H;

R is unsubstituted or substituted, saturated or unsaturated, straight or branched-chain alkyl having from about 10 to about 24 carbon atoms, or $((CH_2)_mO)_p(CH_2)_mW$ where p is an

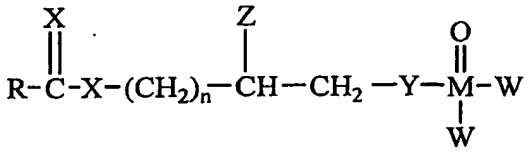
integer from 1 to about 900 and where each m is independently an integer from 2 to about 10 and W is OH, or $O(CH_2)_qCH_3$ where q is an integer from 0 to about 10;

Y is O or S; and

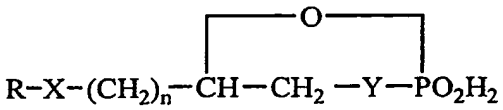
n is an integer from 0 to about 10;

or a pharmaceutically acceptable salt thereof.

89. The composition according to claim 87, wherein the LPA has the formula:



or a cyclic phosphate derivative thereof having the structure:



wherein

X is O, S, or CH_2;

M is P or S, where when M is S, one W is (=O);

each W is independently SH, OH, $OCH_2CH(NH_2)CO_2H$, $OCHCH_3CH(NH_2)CO_2H$, OPO_3H_2, or $OPO_2HOPO_3H_2$, where if one W is OPO_3H_2 or $OPO_2HOPO_3H_2$, the remaining W is OH;

Z is OH, SH, NH_2, halogen, OPO_3H_2, H or SO_3H;

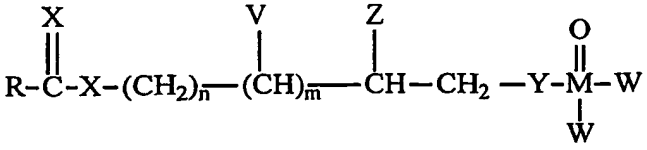
R is unsubstituted or substituted, saturated or unsaturated, straight or branched-chain alkyl having from about 10 to about 24 carbon atoms, or $((CH_2)_mO)_p(CH_2)_mW$ where p is an integer from 1 to about 900 and where each m is independently an integer from 2 to about 10 and W is OH, or $O(CH_2)_qCH_3$ where q is an integer from 0 to about 10;

Y is O or S; and

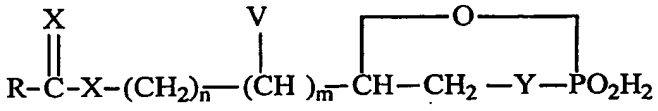
n is an integer from 0 to about 10;

or a pharmaceutically acceptable salt thereof.

90. The composition according to claim 87, wherein the LPA has the formula:



or a cyclic phosphate derivative thereof having the structure:



wherein

each V is independently OH, SH, H, NH_2, halogen, OPO_3H_2, or OSO_3H;

each X is independently O or S;

M is P or S, where when M is S, one W is (=O);

each W is independently SH, OH, $OCH_2CH(NH_2)CO_2H$, $OCHCH_3CH(NH_2)CO_2H$, OPO_3H_2, or $OPO_2HOPO_3H_2$, where if one W is OPO_3H_2 or $OPO_2HOPO_3H_2$, the remaining W is OH;

Z is OH, SH, NH_2, halogen, OPO_3H_2, H or SO_3H;

R is unsubstituted or substituted, saturated or unsaturated, straight or branched-chain alkyl having from about 10 to about 24 carbon atoms, or $((CH_2)_pO)_q(CH_2)_pV$ where q is an integer from 1 to about 900 and where each p is independently an integer from 2 to about 10 and V is OH, or $O(CH_2)_bCH_3$ where b is an integer from 0 to about 10;

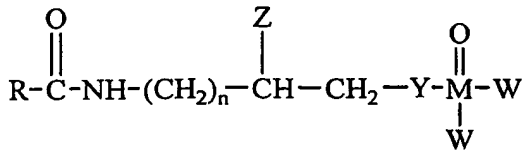
Y is O or S;

n is an integer from 0 to about 10; and

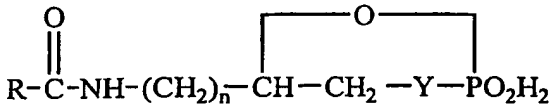
m is an integer from 0 to about 10;

or a pharmaceutically acceptable salt thereof.

91. The composition according to claim 87, wherein the LPA has the formula:



or a cyclic phosphate derivative thereof having the structure:



or the reverse amide thereof, wherein

Z is OH, SH, NH_2, halogen, OPO_3H_2, H or SO_3H;

M is P or S, where when M is S, one W is (=O);

each W is independently SH, OH, $OCH_2CH(NH_2)CO_2H$, $OCHCH_3CH(NH_2)CO_2H$, OPO_3H_2, or $OPO_2HOPO_3H_2$, where if one W is OPO_3H_2 or $OPO_2HOPO_3H_2$, the remaining W is OH;

R is an amino acid side chain unsubstituted or a branched amino acid side chain, or an alkylated amino acid side chain, or substituted, saturated or unsaturated, straight or branched-chain alkyl having from about 10 to about 24 carbon atoms, or $((CH_2)_mO)_p(CH_2)_mW$ where p is an integer from 1 to about 900 and where each m is independently an integer from 2 to about 10 and W is OH, or $O(CH_2)_qCH_3$ where q is an integer from 0 to about 10;

Y is O or S; and

n is an integer from 0 to about 10;

or a pharmaceutically acceptable salt thereof.

92. The composition according to claim 87, wherein the weight ratio of PEG Based Molecule to LPA is 1–100,000 to 1.

93. The composition according to claim 87, wherein the PEG Based Molecule has an average molecular weight from about 8,000 to about 40,000.

94. The composition according to claim 87, wherein the PEG Based Molecule has an average molecular weight of about 20,000.

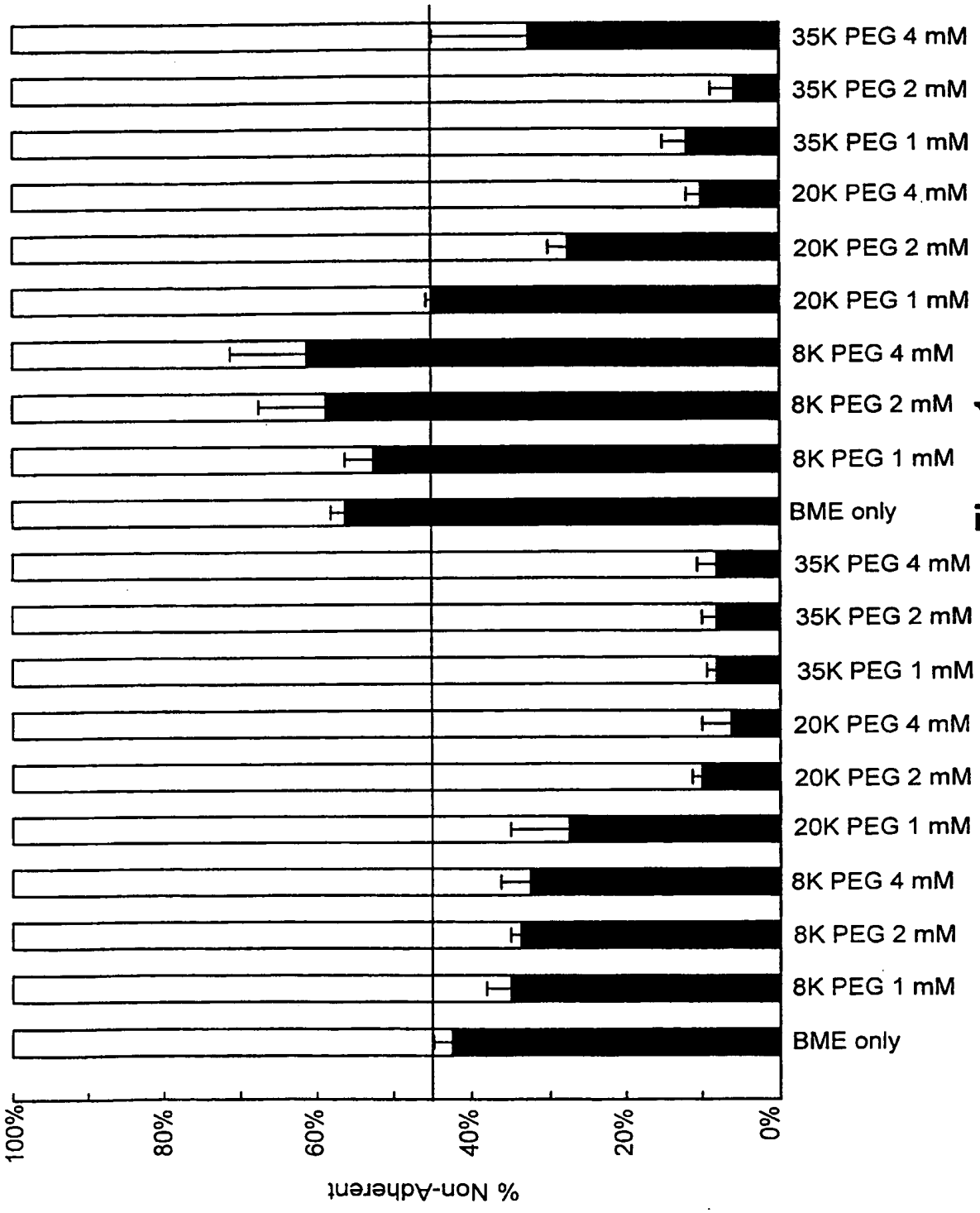

35K PEG 4 mM
35K PEG 2 mM
35K PEG 1 mM
20K PEG 4 mM
20K PEG 2 mM
20K PEG 1 mM
8K PEG 4 mM
8K PEG 2 mM
8K PEG 1 mM
BME only
35K PEG 4 mM
35K PEG 2 mM
35K PEG 1 mM
20K PEG 4 mM
20K PEG 2 mM
20K PEG 1 mM
8K PEG 4 mM
8K PEG 2 mM
8K PEG 1 mM
BME only
100%
80%
60%
40%
20%
0%
% Non-Adherent

Figure 1

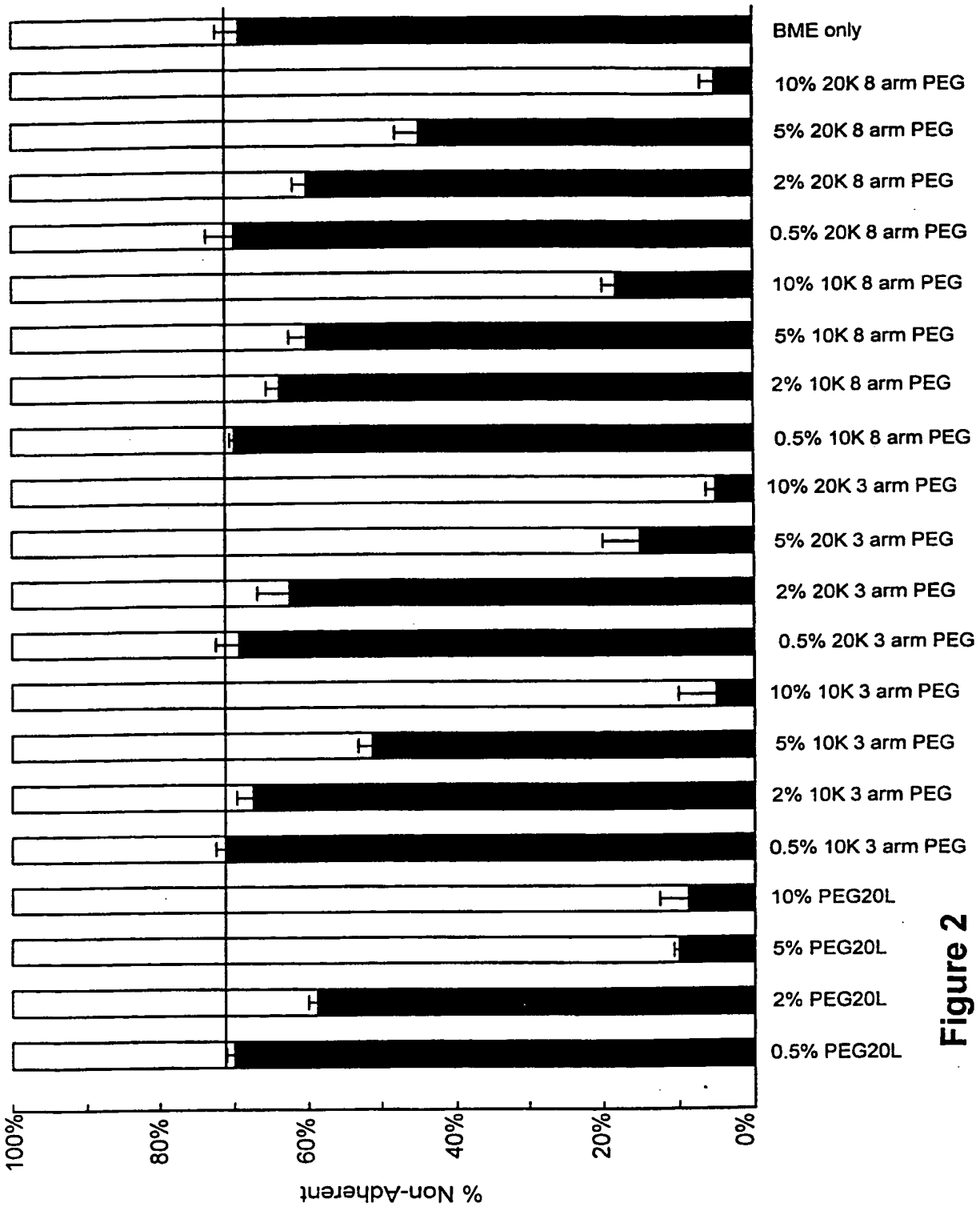

BME only
10% 20K 8 arm PEG
5% 20K 8 arm PEG
2% 20K 8 arm PEG
0.5% 20K 8 arm PEG
10% 10K 8 arm PEG
5% 10K 8 arm PEG
2% 10K 8 arm PEG
0.5% 10K 8 arm PEG
10% 20K 3 arm PEG
5% 20K 3 arm PEG
2% 20K 3 arm PEG
0.5% 20K 3 arm PEG
10% 10K 3 arm PEG
5% 10K 3 arm PEG
2% 10K 3 arm PEG
0.5% 10K 3 arm PEG
10% PEG20L
5% PEG20L
2% PEG20L
0.5% PEG20L
100%
80%
60%
40%
20%
0%
% Non-Adherent

Figure 2

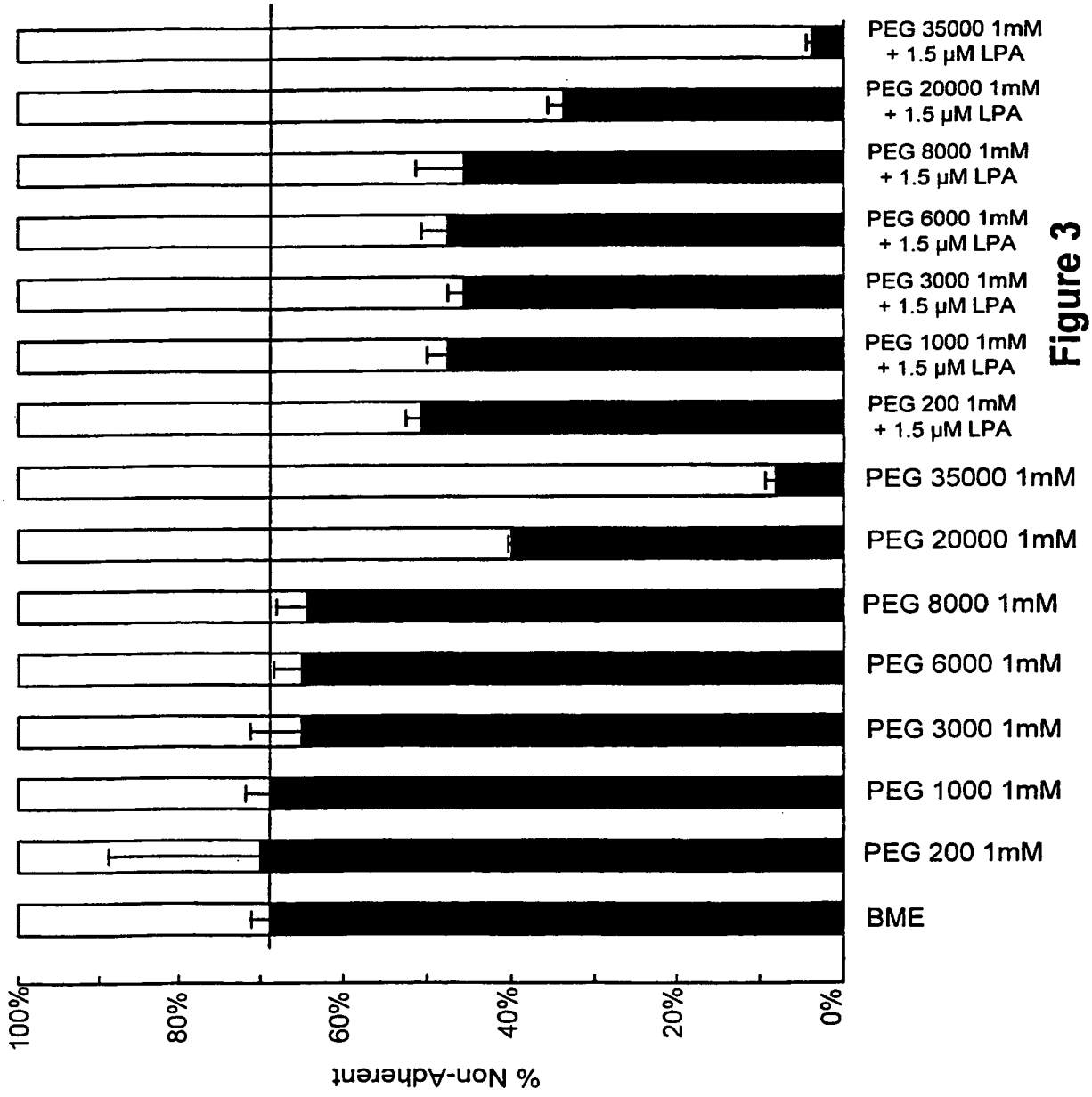

PEG 35000 1mM + 1.5 µM LPA
PEG 20000 1mM + 1.5 µM LPA
PEG 8000 1mM + 1.5 µM LPA
PEG 6000 1mM + 1.5 µM LPA
PEG 3000 1mM + 1.5 µM LPA
PEG 1000 1mM + 1.5 µM LPA
PEG 200 1mM + 1.5 µM LPA
PEG 35000 1mM
PEG 20000 1mM
PEG 8000 1mM
PEG 6000 1mM
PEG 3000 1mM
PEG 1000 1mM
PEG 200 1mM
BME
100%
80%
60%
40%
20%
0%
% Non-Adherent

Figure 3

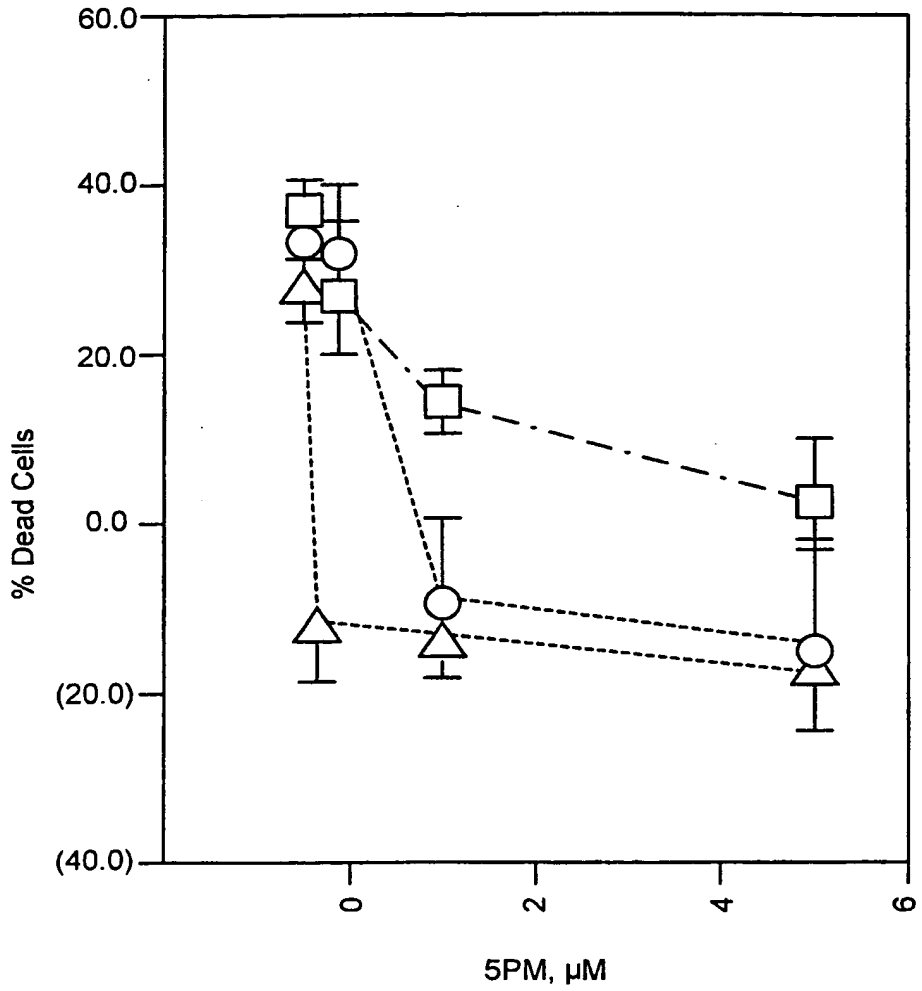


Figure 4

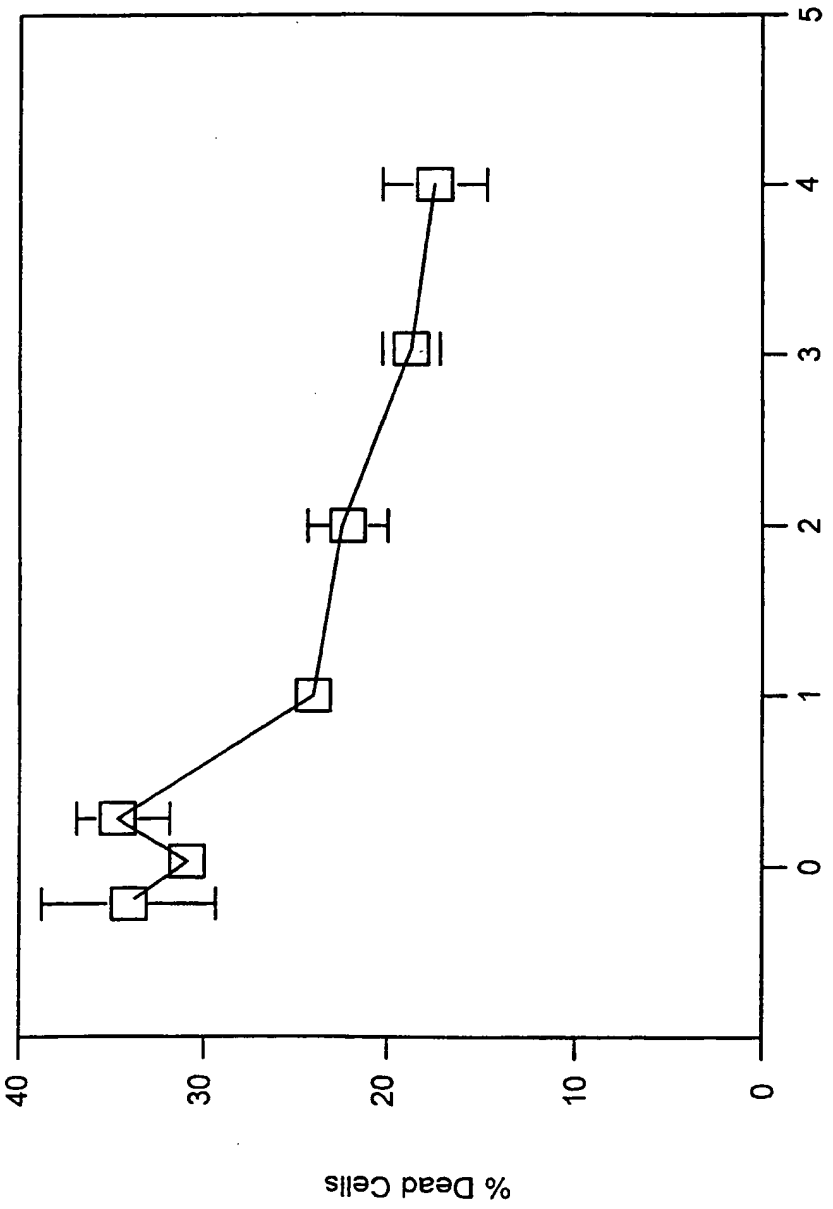


Figure 5

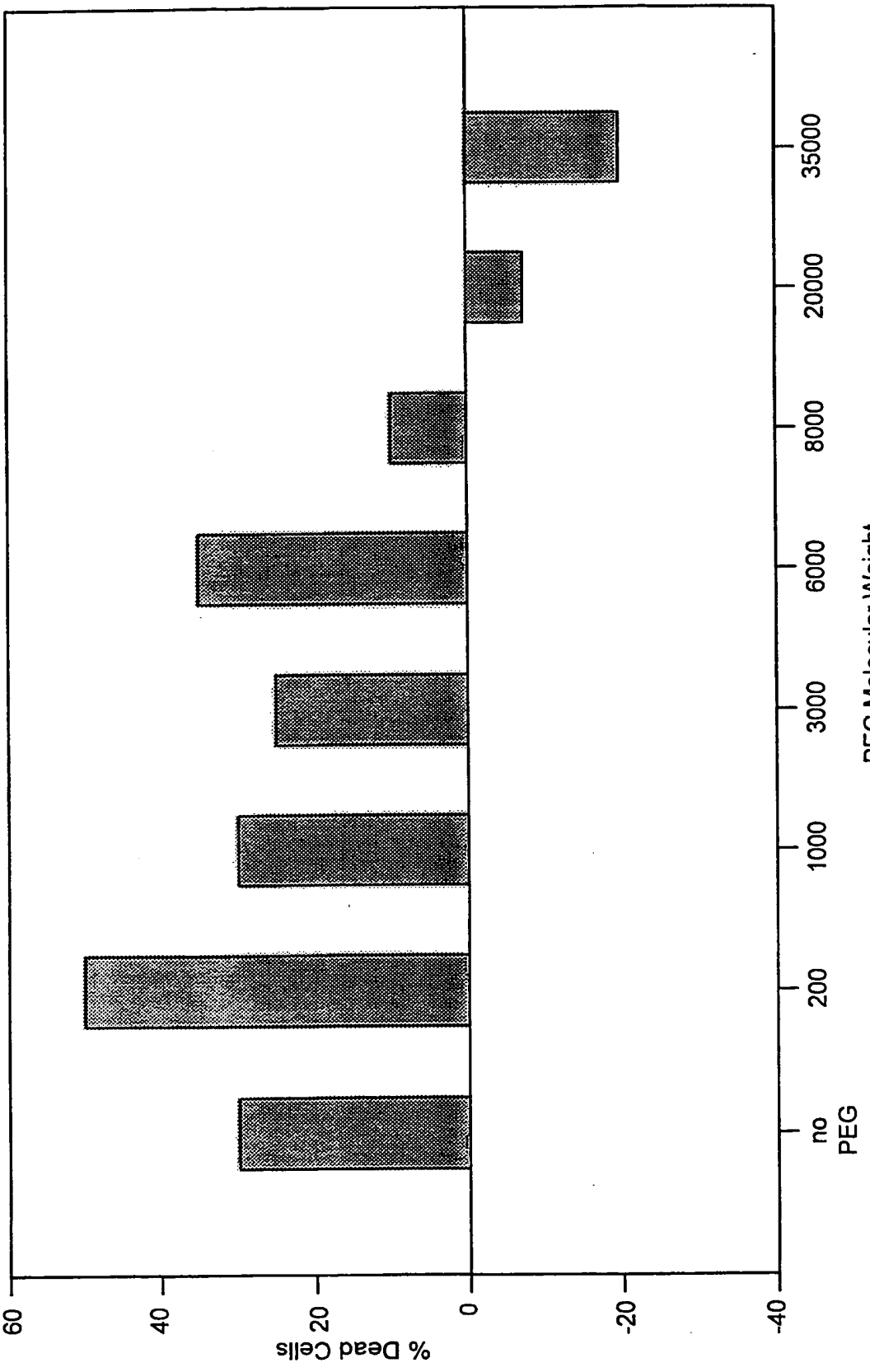

% Dead Cells
60
40
20
0
-20
-40
PEG Molecular Weight
no PEG
200
1000
3000
6000
8000
20000
35000

Figure 6

CONTROL +S, +G

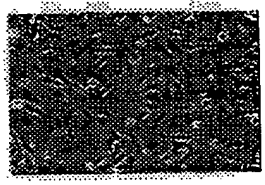


FIGURE 7G

CERAMIDE 10 μM

FIGURE 7A

CERAMIDE 15 μM

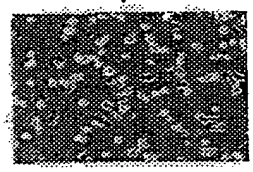


FIGURE 7H

0.3%PEG

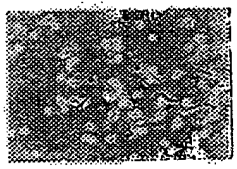


FIGURE 7B

0.3%PEG

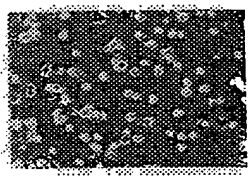


FIGURE 7I

2%PEG

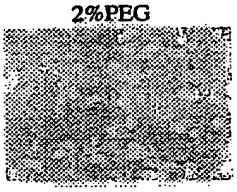


FIGURE 7C

2%PEG

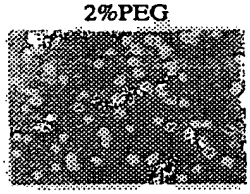


FIGURE 7J

5μM 5PM

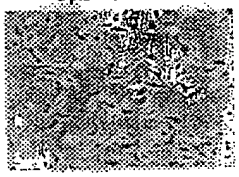


FIGURE 7D

5μM 5PM

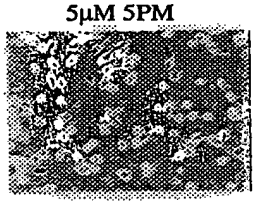


FIGURE 7K

5μM 5PM+0.3%PEG

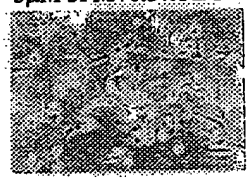


FIGURE 7E

5μM 5PM+0.3%PEG

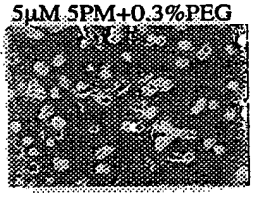


FIGURE 7L

5μM 5PM+2%PEG

FIGURE 7F

5μM 5PM+2%PEG

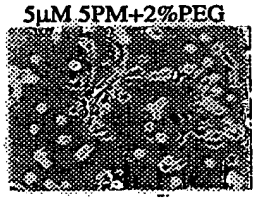


FIGURE 7M

Figure 7A -7M

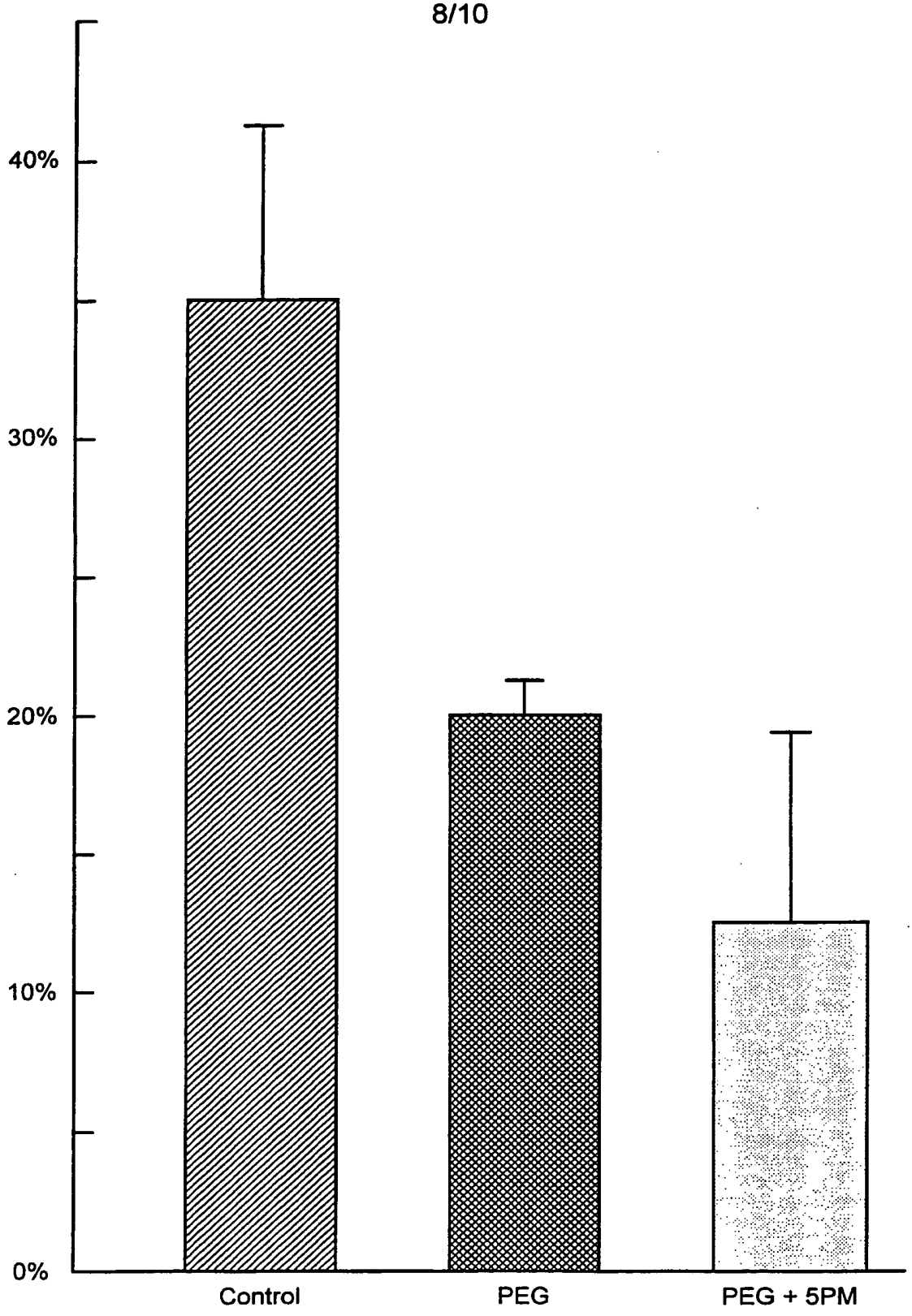


Figure 8

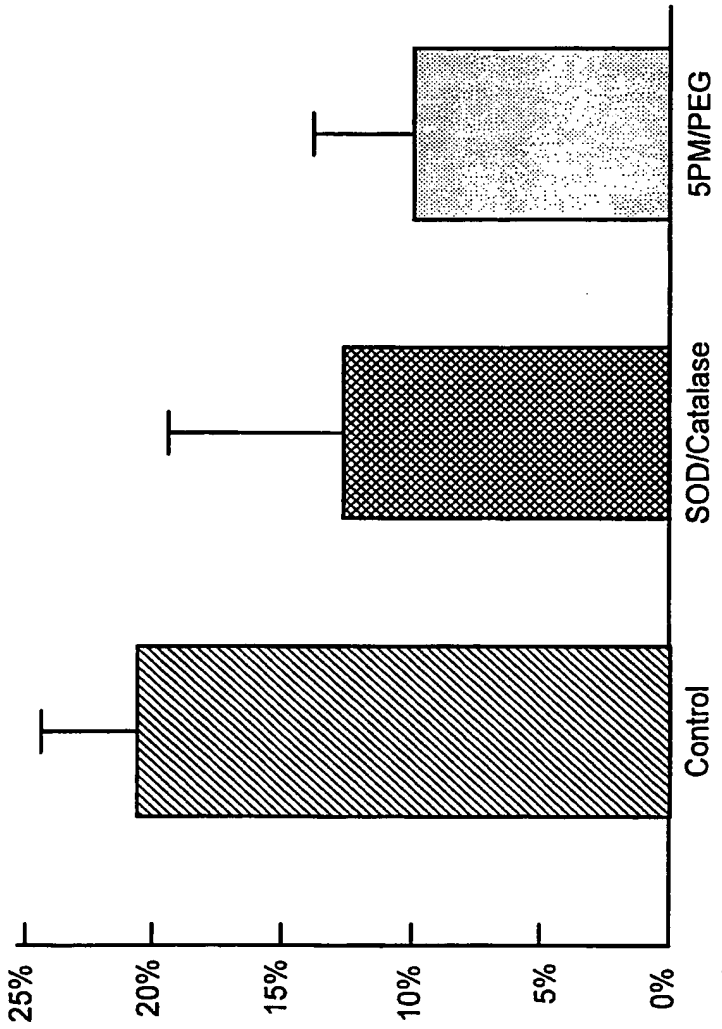


Figure 9

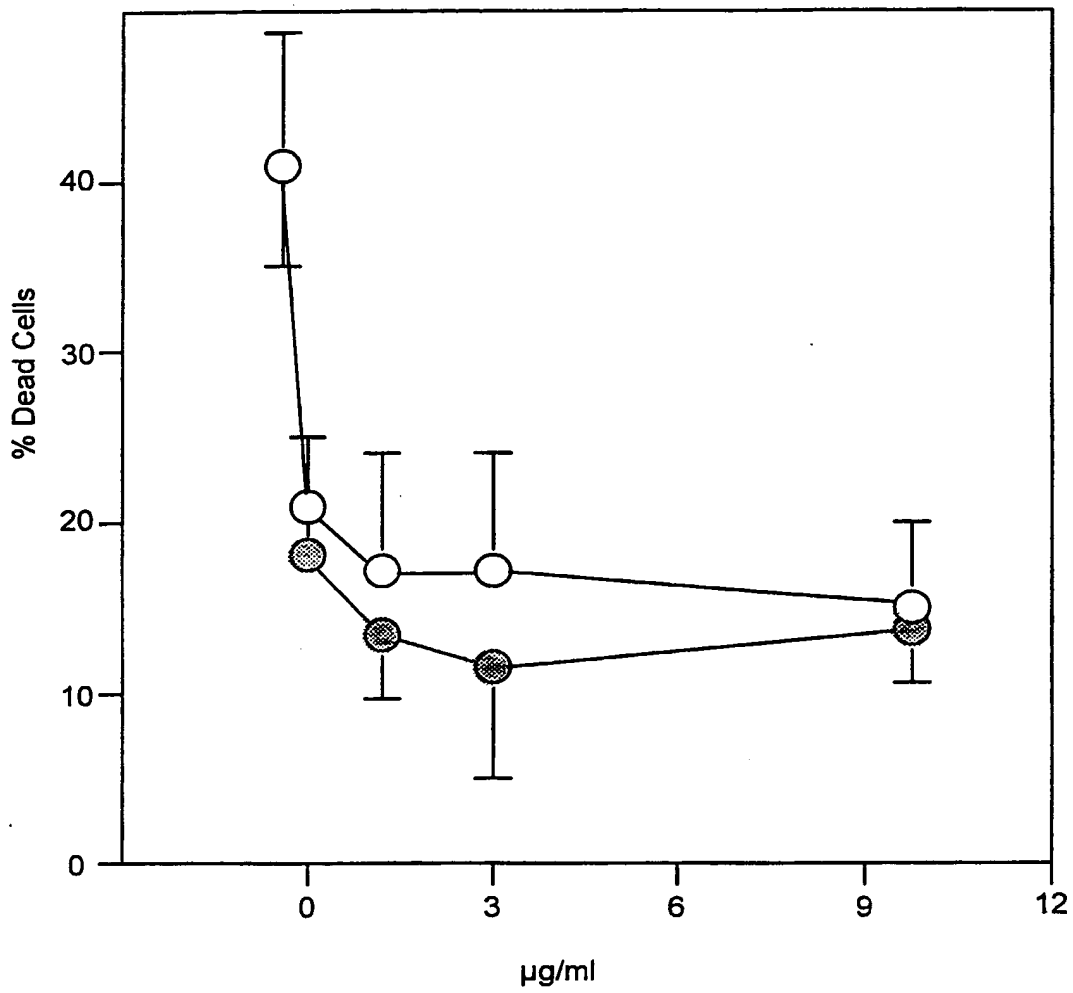


Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17258

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A01N 1/02; A61K 31/765
US CL :424/78.05, 78.06, 78.37
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/78.05, 78.06, 78.37

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,171,264 A (MERRILL) 15 December 1992, see entire document, especially col. 6, lines 8-17.	1-3, 5-6, 8-11, 14-16, 23-24, 29-30
X	US 5,525,635 A (MOBERG) 11 June 1996, see abstract and claim 11.	1-2, 4, 7, 25-27, 29-30
X --- Y	US 5,532,150 A (SNOW ET AL) 02 July 1996, see entire document.	1-5, 7, 17-25, 29-30 ------- 26-28
Y	US 5,624,672 A (BATHURST ET AL) 29 April 1997, see entire document.	17-28
X	WO 86/07535 A1 (FARMACON, INC.) 31 December 1986(31.12.86), see entire document.	1-2, 4, 7, 25-27, 29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
08 NOVEMBER 1998

Date of mailing of the international search report
26 JAN 1999

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Form PCT/ISA/210 (second sheet)(July 1992)*

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International application No.
PCT/US98/17258

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95/09657 A1 (IMMUNO AKTIENGESELLSCHAFT) 13 April 1995(13.04.95), see entire document.	1-2, 4, 7, 29
Y	WO 97/09989 A1 (LXR BIOTECHNOLOGY INC.) 20 MARCH 1997(20.03.97), see entire document.	17-28
X	DANIEL et al. Factors Influencing the Survival of Cell Monolayers during Storage at 4 Degrees. The British Journal of Experimental Pathology. 1976, Vol.57, pages 137-147.	1-7
X ---- Y	NALBANDIAN et al. Pluronic F-127 Gel Preparation as an Artificial Skin in the Treatment of Third-Degree Burns in Pigs. Journal of Biomedical Materials Research. September 1987, Vol.21, No.9, pages 1135-1148.	1-3, 5, 8, 12-13, 17-18, 25-26, 29-30 ------- 28
X	Database Medline on Medline Express Silverplatter 3.11, US National Library of Medicine, (Bethesda, MD, USA), No. 88147910, INGEBO et al. 'Polyethylene Glycol-Electrolyte Solution for Intestinal Clearance in Children with Refractory Encopresis. A Safe and Effective Therapeutic Program,' abstract, Am-J-Dis-Child, March 1988, Vol.142, No.3, pages 340-342.	1-2, 4, 7, 17-19
X	Database Caplus on STN, TAMURA et al. 'Superoxide Dismutase Conjugated to Polyethylene Glycol Provides Sustained Protection Against Myocardial Ischemia/Reperfusion Injury in canine Heart,' Abstract, Circ. Res., 1988, Vol.63, No.5, pages 944-959.	1, 23-24, 29
X	Database Medline on Medline Express Silverplatter 3.11, US National Library of Medicine, (Bethesda, MD, USA), No. 89369536, COLBASSANI et al. 'Modification of Acute Focal Ischemia in Rabbits by Poloxamer 188,' Abstract, Stroke, September 1989, Vol.20, No.9, pages 1241-1246.	1-2, 23-24, 29
X ---- Y	MARSH et al. Hypothermic Preservation of Hepatocytes. I. Role of Cell Swelling,' Cryobiology. December 1989, Vol.26, No.6, pages 524-534.	1-2, 4, 7, 23-24, 29-30 ----- 28
X ----- Y	BANKER et al. 'Freezing Preservation of the Mammalian Cardiac Explant. II. Comparing the Protective Effective of Glycerol and Polyethylene Glycol,' Cryobiology. February 1992, Vol.29, No.1, pages 87-94.	1-2, 4, 7, 23-24, 29-30 ----- 28

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17258

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database Caplus on STN, TEPPLER et al. 'Prolonged Immunostimulatory Effect of Low-Dose Polyethylene Glycol Interleukin 2 in Patients with Human Immunodeficiency Virus Type 1 Infection,' Print Out, J. Exp. Med., 1993, Vol.177, No.2, pages 483-492.	1, 18, 21-22
X	Database Caplus on STN, No. 1994:95322, HOFFMANN et al. 'Protection from Reperfusion-Induced Arrhythmias by Polyethylene Glycol 600,' Abstract, J. Pharm. Pharmacol., 1993, Vol.45, No.12, pages 1093-1095.	1-2, 4, 7, 23-24, 29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/17258

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-30

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17258

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE: POLYETHYLENE-GLYCOLS/THERAPEUTIC USE AND (PRESERV* OR APOPTOSIS OR (CELL DEATH) OR CELL OR CELLS OR TISSUE OR TISSUES OR ORGAN OR ORGANS)
APS: STAR(W)(PEG OR POLYETHYLENE GLYCOL)

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-30, drawn to a method for protecting, preserving or restoring function in cells, tissue, or organs.
Group II, claims 31-53, drawn to a method of treating apoptosis, preserving or restoring function in cells, tissue, or organs.
Group III, claim 54, drawn to a method of preserving or restoring function in cells, tissue or organs.
Group IV, claims 55-70, drawn to a method of preserving or restoring function in cells, tissue or organs.
Group V, claims 71-74, drawn to a method of treating apoptosis, preserving or restoring function in cells *in vitro*.
Group VI, claim 75, drawn to a method of organ preservation.
Group VII, claim 76, drawn to a method of organ preservation.
Group VIII, claims 77-78, drawn to a method of treating cardiac ischemia.
Group IX, claims 79-80, drawn to a method of treating cardiac pathology associated with ST segment shift as measured by electrocardiogram.
Group X, claims 81-84, drawn to a method for protecting, preserving or restoring function in cells, tissue, or organs.
Group XI, claims 85-86, drawn to a pharmaceutical composition for the treatment of myocardial infarction.
Group XII, claims 87-94, drawn to a composition.

The inventions listed as Groups I-XII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The generic invention set forth in claim 1 is taught in Hoffmann et al (1993), among others, and therefore cannot constitute a special technical feature.